CLINICAL PAIN ASSESSMENT IN THE HORSE

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This thesis is dedicated to the memory of Jan Wluka
All my love

CONTENTS

DECLARATION

I declare that this thesis is of my own composition. All assistance and references have been acknowledged.

Rachel A. Eager

14th April 2008

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ABSTRACT

Accurate pain assessment is fundamental to optimal pain management, representing a major welfare concern. Pain assessment has received considerable attention in farm, laboratory and companion animals, however, there is little objective equine pain research. This study aimed to objectively identify behavioural indicators of pain, examining both acute post-operative (castration) and chronic (laminitis) pain.

Male thoroughbred horses (n=10/group) underwent castration or sham castration (control) performed under either standing surgical sedation (SS) or general anaesthesia (GA). Horses were monitored for 24 hours pre-operatively and 48 hours post-operatively. Additionally, seven acute laminitic horses and paired age, sex and breed-matched controls were monitored for up to five days. Assessments were made using time-lapse video recording and direct observation of undisturbed spontaneous behaviour and evoked human interaction behaviours. Data were acquired using The Observer™ and analysed using generalised mixed effects (GME) and discriminant analysis (DA).

GA and SS castrates spent more time with their ears back and displayed a higher frequency of stepping away than controls in interactive tests ($P<0.017$, GME). Head level with withers increased post-operatively in SS castrate, but not control horses ($P<0.001$, GME). Additionally, sham GA resulted in increased inattentive behaviour and hindlimb resting and reduced 'head up' and recumbency ($P<0.039$, GME). Laminitic horses showed reduced hindlimb resting and walking with increased lying, 'head level' and forelimb lifting compared to controls ($P<0.046$, GME). Accuracy of discrimination (DA) between 'painful' and 'pain-free' horses was >78.6% in acute and chronic pain.

We identified behavioural parameters indicative of pain and discomfort in acute and chronic pain states. Acute pain may be most accurately identified through the examination of evoked behaviour, whilst changes in spontaneous behaviour appear more altered in chronic pain.

CHAPTER ONE

INTRODUCTION – ANIMAL PAIN

1.0 INTRODUCTION

'...an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.' (IASP 1979)

As the above definition from the International Association for the Study of Pain implies, pain is a complex phenomenon, combining both physiological (sensory) and psychological (affective) components. Pain provides a protective system, warning of actual or potential damage to the body; discriminating between harmful and harmless situations; allowing escape and withdrawal as well as learning to avoid noxious stimuli or situations (Bateson 1991; Mellor et al. 2000). In this functionality, pain is of significant advantage to the individual, serving a vital protective function. In contrast, when pain continues beyond its useful term, it can become pathological, chronic and debilitating, leading to intense suffering.

Our use of animals within modern society presents a moral and ethical obligation to protect them from suffering (Robertson 2002). Maintenance of animal welfare is a fundamental goal of the veterinary profession (Otto & Short 1998; Paul & Podberscek 2000). If it is assumed that non-human animals experience pain in a similar (but not the same) manner as humans, pain becomes a significant animal welfare concern (Anil 2002).

Assessment of pain severity is of vital importance when considering treatment options. In human medicine, it is often assumed that severity of pain is proportional to degree of injury, yet this is often not the case (Melzack et al. 1982) and it is difficult to generalise on the relationship between tissue damage and pain experienced in conscious human beings (Wall 1992). The physiological responses of the sensory or nociceptive system

can be measured and quantified, and neurophysiological systems involved in pain processing are similar in all mammals (Bateson 2004). Conversely, the emotional experience of pain is subjective, an individual experience, and as such, unquantifiable.

'Pain is always subjective. Each individual learns the application of the word through experiences related to injury in early life.' (IASP 1979)

The definition and recognition of pain in animals has been significantly hindered by a Cartesian reluctance to accept that animals are capable of conscious, emotional experience (Molony & Kent, 1997). Self-report sets the standard for the assessment of pain in conscious human beings, but represents a considerable constraint when considering non-verbal humans or the veterinary patient!

The following review aimed to obtain a greater understanding of animal pain through the study of current literature. With an emphasis on equine pain, this review initially examines the neurophysiology of pain, animal pain and animal welfare. This is followed by an examination of behavioural and physiological changes reported in animals in association with pain for use as possible indicators of pain.

1.1 NEUROPHYSIOLOGY OF PAIN

The understanding of the fundamental neurophysiological mechanisms of pain is crucial to the accurate development of objective pain assessment protocols, development and testing of analgesic agents and the assurance of best practice within the veterinary community. Whilst a detailed review of pain neurophysiology is not considered within the scope of this review, the following sections, present a brief outline of key concepts.

1.1.1 Nociception

Nociception describes the process by which painful stimuli are detected and information regarding these stimuli is transmitted to the central nervous system for processing. Nociceptors are specialised sensory neurons which are activated in response to high-threshold, noxious stimuli (Jensen 2005). Information is then transmitted along the peripheral nerve to second and third order neurons in the central nervous system, which

are interpreted as pain in the conscious brain (Basbaum et al. 2005). In this manner, pain provides a vital tool, informing the organism of actual or potential damage to its tissues, eliciting withdrawal reflexes, promoting healing and recovery and enabling avoidance learning (Bateson 1991; Mellor et al. 2000).

1.1.2 Nociceptors

A variety of somatosensory afferent neurons are present in the body, detecting innocuous stimuli such as pressure, vibration and stretch. However, nociceptors respond only to high-threshold noxious thermal, mechanical and chemical stimuli (Julius & Basbaum 2001). These fibres do not have specialised receptors but terminate in free endings, which are found in most tissues. The cell bodies of nociceptive fibres are mainly found in the dorsal root ganglion (DRG). Depolarisation of the free endings, known as nociceptive terminals, results in the generation of an action potential which is propagated along the axon of the nociceptor.

Nociceptive afferents are primarily Aδ or C fibre neurons, with some Aβ fibres responding preferentially to noxious stimuli. Aδ fibres are either thermal or mechanical nociceptors with a small diameter and thin myelinated. They have a conduction velocity of approximately 5-30 ms^{-1} and are generally associated with a rapid, sharp, 'first' pain. C fibres are non-myelinated and have a slower conduction velocity of about 1 ms^{-1}. These fibres are polymodal, integrating responses to mechanical, thermal and chemical stimluli. These fibres are associated with a longer lasting, dull aching pain (Livingston & Chambers 2000; Basbaum & Jessell 2000). Aβ fibres are fast conducting and myelinated. They respond to innocuous touch and are therefore not pain fibres, however, stimulation of these fibres may reduce pain (Julius & Basbaum 2001).

Nociceptive afferents largely terminate in the dorsal horn of the spinal cord, synapsing with neurons in the marginal layer and substantia gelatinosa of the superficial dorsal horn (Basbaum & Jessell 2000), which mediate withdrawal reflexes and transmit pain signals to the brain. Glutamate is the predominant excitory neurotransmitter mediating synaptic transmission between nociceptive and dorsal horn neurons (Basbaum & Jessell 2000; Julius & Basbaum 2001). This amino acid activates α-amino-3-hydroxy-5-

methylisoxazole-4- propionic acid (AMPA), kainite and N-methyl-D-aspartate (NMDA) receptors.

1.1.3 Physiological and Pathological Pain

Physiological or acute pain occurs in response to injury or damage to tissues and serves a vital short-term protective function, informing the organism of actual or potential damage to its tissues, eliciting withdrawal reflexes, promoting healing and recovery and enabling avoidance learning (Bateson 1991; Otto & Short 1998; Mellor et al. 2000). Physiological pain does not outlast the duration of recovery and generally responds well to analgesia treatment (Molony & Kent 1997).

Pathological pain results from tissue or nerve damage, inflammation and neural dysfunction and is characterised by pain hypersensitivity. Pain continues after recovery and may have no obvious cause (Molony & Kent 1997). Pathological pain can be broadly divided into two classes; nociceptive and neuropathic pains (Basbaum & Jessell 2000).

Nociceptive (also inflammatory) pain results from direct nociceptor activation in association with inflammation and tissue injury. Whilst this type of pain generally responds well to non-steroidal anti-inflammatory drugs (NSAIDs), neuropathic pain is less manageable, and often resistant to opioids (Woolf and Mannion, 1999). Neuropathic pain results from nerve damage or dysfunction, with clinical manifestations including spontaneous pain (often described as stabbing, burning or electric-shock-like), allodynia and hyperalgesia (Woolf and Mannion, 1999). Allodynia refers to the experience of pain in response to an innocuous stimuli such as light touch, where as hyperalgesia is defined as an increased response to a mildly painful stimuli (Basbaum & Jessell 2000).

1.1.4 Peripheral Sensitisation

Peripheral sensitisation occurs when inflamed primary afferent fibres respond to weak and non-painful stimuli (Basbaum et al. 2005). Tissue injury and the subsequent inflammation result in the release of inflammatory mediators. This in turn lowers the

stimulus-intensity thresholds in addition to the direct activation of nociceptive nerve endings. Often known as the 'inflammatory soup', these substances include histamine, cytokines, prostaglandins and growth factors (Julius & Basbaum 2001). Additionally, the release of peptides and neurotransmitters from activated nociceptors facilitates the production of inflammatory mediators from the surrounding non-neural cells and tissue, a process known as neurogenic inflammation (Julius & Basbaum 2001).

1.1.5 Central Sensitisation

Central sensitisation occurs when dorsal horn neurons respond excessively to stimuli from the periphery (Basbaum et al. 2005). Injury or inflammation can result in a sustained nociceptive input from the periphery, causing the release of the neurotransmitter glutamate, which binds to spinal NMDA receptors. The reaction of these receptors with specific synaptic proteins (Husi et al. 2000) and the activation of glial cells in the dorsal horn (Watkins et al. 2001) leads to heightened excitability of the dorsal horn neurons (through the lowering of thresholds for activation) and the increased of receptive fields. A number of studies have found the provision of pre-emptive analgesia prior to a painful stimulus can reduce the effects of central sensitisation (Woolf & Wall 1986; Dickenson & Sullivan 1987; Lascelles et al. 1997).

1.1.6 Endogenous Analgesia

Pain and fear are competing motivational systems which serve different biological functions. Evidence suggests that in situations of fear or stress, pain responses are reduced, producing what is commonly termed 'stress-induced analgesia' (Kavaliers & Colwell 1991). This response has obvious advantages; it allows the animal to minimise signs of distress and therefore reduce the chances of predation, whilst allowing defensive behaviour to take priority (Lester & Fanselow 1985; Sneddon et al. 2003). Stress-induced analgesia has been associated with laboratory-induced stressors such as foot shock (Lewis et al. 1980) and cold water swimming test in rats (Terman et al. 1986). Furthermore, ecologically relevant stressors such as the presence of a predator (Lester & Fanselow 1985; Kavaliers 1988; Blanchard et al. 1991) or an aggressive interaction (Miczek et al. 1982) have been found to reduce pain threshold or pain behaviour. The degree of analgesia appears to be related to the degree of motivation

(Gentle 2001). For example, rats that experienced the introduction of a novel smell into their environment showed some reduction in pain behaviour. However, this reduction in pain behaviour was less than that shown in rats housed next to a predator (Lester & Fanselow 1985).

Alterations in the animal's environment have also been found to alter responses to pain. When placed in an more complex environment, chickens showed significantly reduced pain behaviour in response to sodium urate injection (a model of inflammatory arthritis) (Gentle & Corr 1995; Gentle 2001). These results did not support the possibility of stress-induced analgesia as it was reported that the birds showed no signs of fear. Further work determined that if birds were food-deprived and then given food whilst in pain, they show significantly less pain behaviour than birds not given food (Wylie & Gentle 1998). These changes are thought to be associated with shifting attention focuses. Remarkably, shifts in attention can reduce clinical signs of inflammation, including skin temperature (Gentle & Tilston 1999). In human beings, psychological manipulations such as hypnosis and behavioural modification are commonly used as tools for pain reduction (Jessup & Gallegos 1994; Keefe & Lefebvre 1994). Research suggests that the perceived intensity and unpleasantness of a thermal stimulus increases when attention is directed towards the stimulus and decreases when directed away from the stimulus (Miron et al. 1989).

1.2 ANIMAL PAIN

Definition and recognition of animal pain has been hindered by unwillingness to accept that animals feel pain (Molony & Kent, 1997). The original IASP definition of pain (section 1.0) is fundamentally based on the description of pain experience by the individual. However, inability to provide self-report, as is the case in non-verbal populations such as neonates and animals, excludes these populations from the current definition (Flecknell 1994). Asserting that animals experience pain in a similar manner (but not the same) as human beings, Molony & Kent (1997) have reworked the original IASP definition and it is this definition which is used throughout this thesis when using the term 'pain' in association with a non-human animal.

'...an aversive sensory and emotional experience representing an awareness to the animal of damage or threat to the integrity of its tissues: it changes the animal's physiology and behaviour to reduce or avoid damage, to reduce the likelihood of recurrence and to promote recovery; unnecessary pain occurs when the intensity or duration of the experience is inappropriate for the damage sustained or when the physiological and behavioural responses to it are unsuccessful at alleviating it.'
(Molony & Kent 1997)

Nociceptive activity and severity of injury are not directly related to the perception of pain (Melzack et al. 1982; Wall 1992), thus dividing psychological from physiological components of pain. Whilst the existence of the neurophysiological pain mechanisms in animals, similar to those found in humans, suggests animals may be capable of at least one component of pain, attempting to determine what animals do or do not feel

'...on the basis of a very incomplete understanding of the brain is treading on treacherous ground' (Bateson 2004).

The existence of emotional experience in other human beings is inherently subjective and therefore can never be known, however, is readily assumed. Human beings are able to verbally describe their experience in terms that others understand and can relate to their own thoughts or feelings. The emotional experience of pain is what each individual interprets it to be (Robertson 2002). Pain perception is considered as a phenomenon of the mind and therefore has no physical dimensions (Kitchell 1987). It is not possible to perceive another's pain and/or suffering or be certain that another suffers in the same manner (Bateson 2004). The ability of many animal species to detect noxious stimuli does not directly infer pain perception (Flecknell 2000), which can only be attributed to conscious beings (Livingston 2002).

The Cartesian philosophy prioritised the logical, mathematical components of the physical world. Whilst human beings were attributed subjective awareness due to their communion with God, animals existed as merely mechanical beings. With no capacity for emotion, they were unable to perceive pain and purely produced a mechanical

response to noxious stimuli. The formation of this view into a scientific ideology, only capable of dealing with objective phenomena, removed the question of animal or indeed human consciousness from the scientific arena and alleviated ethical concerns for scientists causing pain to animals (Rollin 1987).

Taking a Darwinian approach that, assumes that pain perception has an evolutionary function in the adaptation to potentially threatening situations and has not just evolved in response to the specific human niche, then it is logical that animals have also evolved pain (Dawkins 1998; Rutherford 2002). Animals show behavioural and physiological responses to potentially painful stimuli, in a similar manner to humans (Dubner & Ren 1999) and there is no reason to suppose that pain perception evolved as a new sensory phenomenon in human beings (Kitchell 1987). The common sense approach usually infers that animals perceive pain (Rollin 1987), although there is an appreciation that is not possible to know with certainty what an animal is experiencing (Mendl & Paul 2004).

The lack of a commonly accepted definition of animal pain does not absolve us of our moral responsibility. Analgesia is administered in human medicine even when there is unreliable and/or subjective quantification of severity (Morton & Griffiths 1985; Rollin 1987).

> *'..we cannot directly perceive thoughts and feeling in animals, we cannot directly perceive quanta or black holes either, all are postulated theoretic entities that are presumed to exist because they provide us with the best explanations for certain phenomena, and enable us to predict features of those phenomena.'* (Rollin 1987)

It is suggested that where there is uncertainty about the existence of pain, animals should be given the benefit of the doubt (Crane 1987; Anil et al. 2002). Animal research is performed on the basis of an analogy with human beings, this analogy should therefore be considered to work both ways: what is painful in human beings should be considered painful in animals (Morton & Griffiths 1985). Judgements on pain levels should be overestimated to avoid overlooking animals in pain at cost of treating some

that are not (Molony & Kent 1997). However, the over-use of analgesic drugs may have detrimental effects on the animal (Flecknell 1994) and treatment of pain may be problematical due to economic, social and legal constraints (Livingston 2002). Pharmacokinetics of analgesic drugs varies widely with species and it cannot be assumed that what is effective in human beings is similarly effective in horses for example (Livingston 2002).

1.3 ANIMAL WELFARE

The protection of animal welfare is a fundamental goal of the veterinary profession (Otto & Short 1998; Paul & Podberscek 2000). In general, social concern for the welfare of animals has been growing, most importantly in the recognition that animals can suffer. On the basis of these assumptions the argument inevitably becomes an ethical debate, suggesting it is ethically unacceptable to ignore animal suffering (Rollin 1987). The determination, however, of what constitutes mistreatment and how to define animal welfare is extremely complicated.

1.3.1 What is animal welfare?

Fraser and Duncan (1997) detail three different concepts, used in the study of animal welfare;

1. feelings-based (defining welfare in terms of subjective or emotional experience)
2. naturalness-based (defining welfare as the ability to perform a full behavioural repertoire)
3. functioning-based (define welfare in terms of normal or satisfactory biological functioning)

Whilst these principles are fundamentally different, they often lead to similar conclusions (Duncan & Fraser 1997). Considering only reproduction and health as indicators of welfare results in relative ease of assessment, however, the consideration of how an animal 'feels' about a situation is more difficult (Mason & Mendl 1993). It may, therefore, be more pertinent to consider welfare as a combination of subjective and objective conditions (Fitzpatrick et al. 2006). Dawkins (2004) suggests that both the

physical and mental aspects of animal welfare are important and that welfare assessments should answer two questions;

'Are the animals healthy?'

'Do the animals have what they want?'

In 1965, The Brambell Committee was formed in order to examine and report on the welfare of intensively farmed animals. The report put forward a set of criteria for maintaining animal welfare, termed 'The Five Freedoms'. These included the freedom to 'stand up, sit down, groom itself, turn round and stretch the limbs' (Brambell 1965). Concerning only a limited number of maintenance behaviours, these criteria were inadequate (Webster 2004). The evolution of these concepts has led to the current 'Five Freedoms' described by the U.K. Farm Animal Welfare Council in 1992;

1. Freedom from hunger, thirst and malnutrition.
2. Freedom from discomfort.
3. Freedom from pain, injury and disease.
4. Freedom to express normal behaviour.
5. Freedom from fear and distress.

1.3.2 Pain and equine welfare

As a complex phenomenon, combing both physiological and psychological components, pain may negatively influence equine welfare in many ways. Physiological factors may affect the functioning of the animal, whilst the psychological, emotional elements may result in negative feelings.

From a functional perspective, pain can induce haemodynamic, respiratory and endocrine changes resulting in tissue ischaemia, tissue hypoxia, shock, cardiac arrhthymia and renal failure (Otto & Short 1998). These changes may in turn result in the induction of catabolism, delaying wound healing, prolonging hospitalisation and increasing morbidity and mortality (Otto & Short 1998; Flecknell 2000). Chronic pain can be debilitating and fatiguing (Crane 1987), with possible impairment of immune

function, especially in natural killer (NK) cell function. These cells defend the body against viral and bacterial infection and help inhibit tumour growth (Page 2005).

If we assume that an animal is capable of suffering in a similar manner to human beings, pain is a noxious emotional experience which may lead to depression, withdrawal and social isolation (Molony & Kent 1997). The emotional experience of pain is inherently subjective and therefore often overlooked by veterinarians and scientists taught only to examine objective data and measurable responses (Rollin 1997).

Examining the effects of pain from a 'naturalness' point of view, pain may result in behavioural restriction. For example pain-related lack of mobility may result in reduced food and water consumption (Flecknell 2000).

Pain is a significant welfare issue, whichever definition or concept of welfare is considered. Freedom from pain is included in the 'Five Freedoms' and pain can be shown to negatively influence welfare in the functioning, feelings and naturalness concepts. Pain represents a significant welfare concern when applied to both questions posed by Dawkins (2004). As previously mentioned, pain has obvious adverse health effects. Additionally, animals have been shown to be highly motivated to avoid pain and will self-administer analgesic drugs (Kupers & Gybels 1995; Danbury et al. 2000; Colpaert et al. 2001) if given the opportunity, suggesting that they '*want*' to avoid or minimise pain.

Our use of the horse in modern society, in sport, in competition, as food and as a companion animal implies that we have an ethical obligation to protect them from pain and suffering (Robertson 2006). The need for reliable and accurate protocols for the assessment of animal pain is therefore fundamental for the improvement of equine welfare. Only once this has been achieved can we begin to accurately evaluate analgesic agents, refining analgesic protocols, and reliably determine start and end points for analgesic therapy (Flecknell 1994; Flecknell & Roughan 2004). Effective monitoring will allow flexibility in dose regimes, tailoring analgesic therapy to the individual needs

of the animal, limiting both over- and under dose. Improved pain assessment techniques will also enable the objective comparison of different techniques for procedures such as castration, allowing modification and identifying *best practice*.

1.4 PAIN ASSESSMENT IN ANIMALS

Pain assessment in human beings represents a considerable challenge to the medical profession due to the enormous variation in subjective responses to standardised stimuli (Bateson 1991). In animals and non-verbal human beings, this is further complicated by an inability to provide a self-report of perceived pain severity. In these situations, approaches to pain assessment involve a value-judgement based on measurable behavioural and physiological changes indicating pain-induced stress (Molony & Kent 1997). Estimations of pain severity may be based on magnitude of behavioural and/or physiological changes, interference with normal behaviour and through analogy with experience similar treatments in humans (Molony et al. 2002). The validation of these indices and their response to pain severity is difficult as no 'gold standard' exists against which changes can be measured (Molony et al. 2002); existing indices giving only an indication of level of negative experience (Mellor & Stafford 1999). Without an adequate method of pain assessment it is necessary to assume a similar level of pain between animals and human beings. But considering differences in anatomy and behaviour this is unlikely to be the case (Flecknell 1994).

1.4.1 Problems in animal pain assessment

The characteristics of observer and patient may affect animal pain assessment. Whilst it is widely accepted that different species may respond to pain in different ways, pain tolerance and expression may vary with motivation, stress, previous experience, breed, location and severity (Kitchell 1987; Matthews 1992; Archer et al. 2004). Species and breed difference make extrapolation difficult (Benson & Thurmon 1987; Archer et al. 2004). In some, particularly prey, species, stoical behaviour may confound pain assessment (Crane 1987). Other species may show clear behavioural responses to pain, eliciting help from others. However, in species such as the horse, overt pain behaviour would signal reduced fitness and hence easy prey (Flecknell 2000). The natural history of the horse has specified a good memory for noxious stimuli and a flight response to

threatening situations. Horses may be reacting to stimuli that are presently painful but also may be performing patterns of behaviour that have been learnt to avoid painful situations (Casey 1999). Pain may also be influenced by previous experience and current social position (Fitzpatrick et al. 2006).

There may be a number of significant influences on an observer's judgement of animal pain severity. Anthropomorphism is the attribution of human characteristics to animals or inanimate objects and may affect judgement in a number of ways. Firstly, those animals that are thought to possess human characteristics (such as smiles in dolphins) may be given greater consideration than those that are perceived as 'nasty' (Wall 1992). Empathetic feelings towards an animal may confuse what the *animal* is actually feeling with what the *observer* is feeling (Anil et al. 2002). Furthermore, the failure of an animal to react to a painful stimulus in a manner similar to that of a human may result in the conclusion that significant pain is not being experienced. Traditional notions of breed characteristics may affect both pain assessment and treatment (Holton et al. 1998), a problem which is especially prominent in equines where native breeds such as the Shetland pony are considered more hardy than warmblood breeds such as the Thoroughbred (Taylor et al. 2002b). Additionally, attribution of pain scores, for example, may be significantly affected by the observer's experience of animal pain (Anil et al. 2002).

In equine veterinary medicine, there is a lack of consensus in the attribution of pain levels to specific procedures (Price et al. 2002), highlighted by debate in the veterinary press regarding the provision of analgesia following equine castration (Capner 2001; Johnson 2001; Harris 2001; Jones 2001; Flecknell et al. 2001; Green 2001). Consequently, importance is attributed to preconceived perceptions of the relationship between pain severity and particular procedures (Holton et al. 1998).

1.4.2 Clinical pain assessment

Tools for the assessment of pain in a clinical setting must be practical, reliable and sensitive. Training and experience should improve observer assessment (Molony & Kent, 1997). Simple criteria needed in order to improve the efficacy of the training of

veterinary staff (Cambridge et al. 2000). Assessment may be improved by a deeper knowledge of species, breed and of the individual animal (Molony & Kent 1997). However, in a clinical or farm setting this may not be possible.

Numerous scales and scores have been developed for the assessment of animal pain. However, few of these have been satisfactorily evaluated (Flecknell & Roughan 2004). These commonly omit to control for factors such as effect of drugs, and they often including highly subjective assessment criteria. Pain scores such as the Obel grading scale for laminitis (Obel 1948) are regularly used in equine practice. Again, these scales are frequently employed without prior validation. Composite scores, including a number of variables may help reduce inter-observer variation. However, a lack of validation of the score in general and of individual parameters reduces their application and efficacy. The current, lack of knowledge of pain assessment in animals limits the practical use of these scores (Flecknell & Roughan 2004). In order to optimise the development of such scores, the validity of sensitivity of each parameter must be determined and redundant indices must be eliminated (Molony & Kent 1997). A further discussion of subjective pain scores can be found in section 1.5.1. It is also vital that a clinical pain assessment protocol is capable of distinguishing between the effects of pain and those of anaesthetic and analgesic drugs used (Firth & Haldane 1999), as over-dosing can be detrimental to health (Flecknell 1994). It should be recognised that responses to drugs may vary significantly between species (Benson & Thurmon 1987).

1.5 SUBJECTIVE AND OBJECTIVE ASSESSMENT TECHNIQUES

Pain assessment in human subjects is fundamentally subjective, as it evaluates pain experience on the basis of verbal description (Sanford 1992). However, as previously discussed, this is not possible in veterinary medicine, and whilst assessment of indicators of pain is often categorised as subjective or objective (Hansen 1997), all assessments of animal pain are based on observer interpretation and therefore they are subjective in nature.

'When you can measure what you are speaking about, and express it in numbers, you know something about it; but when you cannot measure it, when you cannot express it

in numbers, your knowledge is of a meagre and unsatisfactory kind; it may be the beginning of knowledge, but you have scarcely in your thoughts advanced to the state of Science, whatever the matter may be.'(Kelvin 1883)

Kelvin's argument suggests it is difficult to give a scientific account of subjective emotional experience, and therefore pain, in animals. There is no objective 'gold standard' behavioural or physiological variable that can be measured to determine pain severity or against which potential indicators of pain can be titrated (Molony 1992). The observation of behavioural indicators of pain has traditionally been described as subjective with an overall assessment of pain severity gained through whole animal subjective observation. However, this type of assessment is associated with problems of repeatability and reproducibility and may be severely affected by the individual perceptions and beliefs of the observer. Objective parameters were only considered as physiological variables such as heart rate that could be easily measured. More recently the objective examination of behavioural parameters using techniques such as quantitative sensory testing has objectified behavioural pain assessment. Tools for subjective and objective assessment of pain are described in the following sections.

1.5.1 Subjective Assessment

Pain scales are commonly used in human medicine as an *aide-memoir*, allowing patients to report on their progress (Wall 1992). Frequently, scales used in veterinary medicine are simply extrapolated from use in human medicine without consideration for validity and accuracy (Flecknell 1994; Holton et al. 2001). Arbitrary criteria for assessment are used on the assumption that they signify pain without prior validation.

Whilst subjective assessments are quick and easy to use and allow the observer to consider the state of the animal as a whole, as previously discussed many extraneous influences may affect observer judgement. Both uni- and multi-dimensional scales have been applied for the assessment of pain in both human and veterinary medicine. These tools are described and discussed below. Qualitative techniques have recently been developed for the assessment of animal welfare, which use free-choice profiling

(Wemelsfelder et al. 2001). However, these techniques have not yet been applied to the assessment of pain.

1.5.1.1 Unidimensional scales

Unidimensional pain scales are the most commonly used in veterinary medicine (Holton et al. 2001). Typical examples include the simple descriptive scale or SDS and numerical rating scale or NRS, which are based on a 4-5 point scale using descriptive terms (SDS) or numbers (NRS) to rate severity and are shown in figures 1.1 and 1.2. The visual analogue scale (VAS) consists of a 10 cm line representing a continuum from minimum to maximum (see figure 1.3). Observers are required to place a mark on the line to indicate perceived severity.

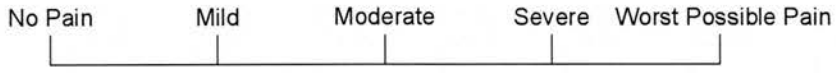


Figure 1.1 A simple descriptive scale for the assessment of pain.

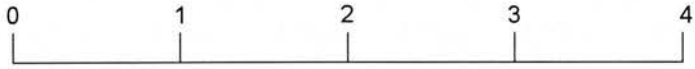


Figure 1.2 A numerical rating scale for the assessment of pain.

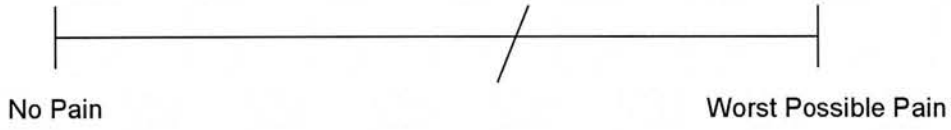


Figure 1.3 A visual analogue scale for the assessment of pain.

These scales have been used in a number of studies of animal pain. For example, the scale can be used to compare the efficacy of post-operative analgesia in dogs (Reid & Nolan 1991; Nolan & Reid 1993), and for the assessment of lameness in cattle (O'Callaghan et al. 2003). The NRS is most frequently used in equine veterinary practice for the assessment of lameness (Fuller et al. 2006).

These scales can be used quickly and easily, making them applicable in many settings (Chapman et al. 1985). However, care should be taken to examine inter- and intra-

observer reliability to determine the most appropriate scale. Although simple to use, both NRS and SDS scales lack sensitivity, as it is impossible to record small changes in pain severity (Reid & Nolan 1991; Welsh et al. 1993; Holton et al. 1998; Firth & Haldane 1999). An assumption is also made that each division represents an equal increase or decrease in the pain experienced (Welsh et al. 1993). The use of a continual line in the visual analogue scale, may prevent the forced grouping of unlike data, with measurement unconstrained by the addition of units (Welsh et al. 1993). The accuracy of the VAS may be affected by visual acuity and motor coordination (Holton et al. 1998; Firth & Haldane 1999). Welsh et al. (1993) found reproducibility to vary along the line, with optimal reproducibility occurring at the either end. Additionally, it has been suggested that the expression of a broad range of experience may result in observers spreading scores over the entire scale, regardless of the magnitude of sensation (Chapman et al. 1985). Clinical lameness grading scores cover an extremely wide variation, from sound to horses not being able to bear weight. It has been suggested that these scales may not be sensitive enough to detect clinically relevant differences in severity (Keegan et al. 1998). These types of scales all rely on the observer's ability to empathise with the animal and are therefore easily confounded by differences in personality, beliefs, mood and past experience (Sanford et al. 1986). These factors may all reduce the reliability and sensitivity of the scoring technique (Rutherford 2002).

1.5.1.2 Multidimensional (composite) scales

Unidimensional scales only assess the intensity of pain experience, however, pain is a complex multifactorial phenomenon, which may be described in many different ways, expressing a variety of aspects of pain experience (Melzack & Katz 1999). Descriptions such as burning, stabbing, itching and cramping and other endless qualities of pain experience are not be identified by a unidimensional scale. The McGill Pain Questionnaire (Melzack 1975) is possibly the most famous example of a medical multidimensional pain assessment tool. This system requires patients to grade pain experience on the basis of a wide variety of terms, giving information on the sensory, affective, evaluative and temporal aspects of pain. In animals, signs of pain may not all be present at the same time or relate to the same components of pain experience

(Morton & Griffiths 1985). In recognition of the multidimensional nature of animal pain a number of pain assessment tools have been developed, observing a number of behavioural and physiological parameters in combination (Morton & Griffiths 1985; Raekallio et al. 1997a; Raekallio et al. 1997b; Firth & Haldane 1999). However, these scales are often used without consideration of sensitivity, reliability and validity.

1.5.2 Objective Assessment

Objective assessment requires a measurement to be made. In the case of physiological variables such as heart rate, the assessment is easy. However, when examining behavioural variables observers must examine the duration and frequency of behaviour. The pursuit of objectivity in the assessment of behavioural parameters has led to the development of a number of different tools for the objective identification of changes in behaviour. These include (as discussed below) quantitative sensory testing for the study of nociceptive thresholds and hyperalgesia; gait analysis for the assessment of lameness and force plate analysis, examining changes in weight-bearing and limb loading.

1.5.2.1 Quantitative Sensory Testing

Quantitative sensory testing (QST) involves the objective measurement of response to standardised noxious stimuli. Testing involves the application of thermal, mechanical or electrical stimuli, which can be increased in intensity, eliciting a withdrawal response. Most commonly latency to respond is recorded. Originally developed for the assessment of nociceptive sensory function and hyperalgesia in human beings and laboratory animals, QST techniques have now been successfully developed for use in sheep (Ley et al. 1989; Welsh & Nolan 1995), cattle (Ley et al. 1996; Whay et al. 1997; Veissier et al. 2000), dogs (Lascelles et al. 1997), cats (Rice & Kenshalo 1962) and horses (Pippi & Lumb 1979; Kalpravidh et al. 1984a; Kalpravidh et al. 1984b; Moens et al. 2003).

In the horse, the majority of experimental studies have used thermal, mechanical and electrical stimuli to examine analgesia in clinically normal animals (Pippi & Lumb 1979; Kalpravidh et al. 1984a; Kalpravidh et al. 1984b; Moens et al. 2003). In equine clinical practice, the application of hoof testers and flexion tests represent clinical forms of QST. However, the assessment of such tests is subjective. Thermal stimuli are most

commonly created through the focusing of a light beam on to the coronary band, with end points such as foot lifting (Kalpravidh et al. 1984b) detected by an accelerometer (Pippi et al. 1979). The response to the application of a mechanical stimulus, gradually increasing in pressure, has been used to study hyperalgesia in a number of farm animal species (Fitzpatrick et al. 2006). In the horse, Moens et al (2003) used a pneumatically operated pin pressing on canon bone (with foot lifting as an end point) to test the antinociceptive effects of three analgesic agents. Von Frey filaments are calibrated to deliver a specific force on the skin, allowing quantification of the force required to elicit a response and have been used to assess the effect of pre-emptive ketamine on wound sensitivity in the horse (Rédua et al. 2002). Further studies have measured *'hoof compression threshold'* (Owens et al. 1995), objectifying traditional techniques for the assessment of hoof pain. Electrical stimuli are less regularly used, but the use of a gradually increasing DC current, applied to the coronary band (foot lifting end point) has been used in horses (Moens et al. 2003).

When extrapolating QST data to broader situations it must be remembered that pain experience cannot be referred from reflex action alone (Lascelles et al. 1997). It is also impossible to determine whether or not the animal is reacting before or after, or at the exact time at which the stimulus becomes painful (Conzemius et al. 1997). Welsh and Nolan (1995) reported increased variation in responses to mechanical threshold testing in 'naïve' animals (those unfamiliar with the testing protocol). These results suggest that the performance of a reproducible test requires that animals must be trained prior to testing. This would limit the application of QST techniques in clinical practice. Furthermore, a number of technical problems may constrain the use of QST. These include significant alterations when more than one operator is used, and the influence of environmental variables such as air temperature (Siao Tick Chong & Cros 2004).

The increased threshold to noxious stimulation has been associated with stress, alterations in mental state and the diversion of attention (Siao Tick Chong & Cros 2004). Stress, environment and attentional functions have all been associated with the reduction or elimination of pain behaviour (Gentle 2001). In terms of alterations in nociceptive thresholds, studies examining a variety of laboratory stressors have

identified significant stress-induced analgesia in rats (Lewis et al. 1980; Amit & Galina 1986). Furthermore, exposure to ecologically relevant aversive stimuli, such as those associated with predation (Kavaliers 1988; Blanchard et al. 1991; Kavaliers & Colwell 1991) and aggressive encounters (Miczek et al. 1982), have been shown to increase nociceptive thresholds in laboratory and wild rats and mice, indicating endogenously-modulated stress-induced analgesia. In human beings, perceived intensity and unpleasantness of a thermal stimulus was increased when attention was directed towards the stimulus and decreased when directed away from the stimulus (Miron et al. 1989). Additionally, level of alertness in monkeys has been associated with alterations in pain thresholds (Hayes et al. 1981). Clinical use of QST would therefore require horses to be fully habituated to their surroundings in order to reduce environmental effects. The use of a portable system, similar to those developed for dogs (Lascelles et al. 1997) may enable testing to be performed in the home environment. Horses should also be given time to habituate to the testing procedure in order to avoid the possible generation of stress-induced analgesia.

Previous conditions may also affect the response to QST. For instance it has been shown that alterations in mechanical thresholds persist for up to three weeks following clinical resolution of 'footrot' *(Bacterioides nodosues* and *Fusobacterium necrophorum* infection) in sheep (Ley et al. 1989). In instances where an animal's full clinical history is unknown, as often occurs in welfare cases and in chronic conditions, the clinical application of the tests may be constrained. It should also be remembered that individual variation in pain thresholds are often large. Thus results may vary with site and type of test used, and this level of variation may be differ between species (Pippi et al. 1979; Siao Tick Chong & Cros 2004).

Pain sensation cannot be inferred from reflex alone (Chapman et al. 1985) as changes in reflex can result from motor and sensory processing. Whilst QST techniques are often cited as 'non-invasive'(Siao Tick Chong & Cros 2004), serious consideration of the effects of the repetitive application of noxious stimuli on animal welfare should be taken into account.

1.5.2.2 Kinematic Gait Analysis

Kinematics is the study of the changes in position of particular parts of the body in a specified time frame and is measured in terms of linear and angular parameters (Barrey 1999). Markers (such as white spots or reflective stickers) are fixed at standardised anatomical positions. During movement, successive images are recorded and then analysed for movement of the markers. Initially this work was based on chronophotography. However, more recent techniques have used digital video recording and computerised-tracking of markers (Flower et al. 2005).

Evaluation of equine lameness is traditionally performed through the visual observation of movement and recorded on an officially accepted NRS grading system (Stashak 1987). However, as previously discussed in section 1.5.1, these systems may not be sensitive enough to detect small but clinically relevant, changes in lameness (Keegan et al. 1998) and may result in substantial variability (Keegan et al. 2003). Objective kinematic techniques have been developed and used extensively in the equine industry. These tools examine fundamental gait characteristics, the effects of velocity and incline and changes in gait as a result of training (Barrey 1999). Kinematic techniques have been effectively used to objectively identify and characterise irregularities in gait associated with naturally occurring (Peham et al. 1999; Peham et al. 2001) and induced (Kelmer et al. 2005) lameness. Alterations in vertical head movement have been associated with forelimb lameness in the horse, with the head lowering during the stance phase of the sound limb and raising as the lame limb lands (Vorstenbosch et al. 1997). Variation in stride length was also found to decrease in association with forelimb lameness. This pattern that was reversed following regional anaesthesia (Peham et al. 2001). Similar changes in gait have been associated with sole ulcers or chronic foot lesions, in dairy cows (O'Callaghan et al. 2003; Flower et al. 2005).

Due to the minimal need for handling, kinematic techniques can provide a non-invasive method off assessing movement (Flower et al. 2005). However, accuracy may be reduced by skin displacement or deviation from a parallel place with the camera and care must be taken in the standardisation of markings (Corr et al. 2003). Practically, the

high cost and technical maintenance may reduce the viability of kinematic systems in clinical practice (Barrey 1999; Taylor et al. 2002b).

1.5.2.3 Force Plate Analysis

Locomotion creates forces that are transmitted to the ground (Corr et al. 2003). During limb pain, loading of the limbs and therefore ground force may vary. A plate, which is fitted into the floor or runway, records the ground forces exerted as animals move across it. Using four individual force plates, Hood et al. (2001) examined limb loading in horses with acute and chronic laminitis. The findings suggest that whilst loading is similar to controls in acute laminitics, forelimb loading is significantly reduced in chronic cases. Force plate technology has also identified abnormal loading patterns in horses with navicular syndrome, which were reversed upon nerve blocking (Williams 2001). In addition to the characterisation of disease-associated changes in gait, force plate technology has been used to determine efficacy of treatment; for example following therapeutic shoeing in laminitis patients (Taylor et al. 2002a) and treatment with analgesic agents in horses with navicular (Erkert et al. 2005).

As an objective tool for the assessment of limb loading and gait parameters, force plate analysis provides clear, objective data. The increased sensitivity of the technology detected changes in laminitic horses that were not possible to monitor using the subjective Obel or clinical grading system (Taylor et al. 2002a). Force plate analysis may therefore provide a useful, objective tool for the detailed monitoring of chronic conditions and the assessment of therapeutic efficacy. However, as with gait analysis systems, the use of high cost, large scale technology may limit the clinical applicability of force plate analysis, although the development of 'in-shoe' models may provide a more useful tool (Taylor et al. 2002b). Changes in gait may occur in response to a wide variety of disorders, generally in a non-specific manner and with a wide individual variation. This may affect its use as a diagnostic tool (van Weeren 2002). It should be remembered that changes in gait may be associated with disease-related mechanical changes and therefore may not be solely pain-related (Barrey 1999; Hood et al. 2001).

1.5.3 Summary

Subjective assessment of pain is most the commonly used method of assessment in equine practice. However, it may show reduced reproducibility and repeatability in response to many observer influences. Objective behavioural research in equine pain is sparse. Sources are frequently anecdotal or based on clinical experience. Most studies examine a small number of pre-selected behaviours, which are often chosen without adequate validation. Characterisation of equine pain behaviour through scientific study has been restricted by the tendency to aggregate a number of parameters to form a composite score (Raekallio et al. 1997a; Raekallio et al. 1997b; Pritchett et al. 2003) and by failing to reporting exact frequencies and/or durations of behaviours assessed. This means that it is impossible to determine which behaviours are having the greatest effect and to assess their reliability as biomarkers of pain. Only one study (Price et al. 2003) as yet has examined a wide range of behaviours without prior judgement.

1.6 BEHAVIOURAL INDICATORS OF PAIN IN HORSES

Clinically, behavioural parameters are most commonly used to assess pain in animals. Molony & Kent (1997) classify behavioural responses to pain into four different categories;

1) responses that allow behavioural modification through learning to reduce the possibility of further injury
2) responses that protect parts of the body from injury and which are often reflex
3) responses that minimize pain and encourage healing
4) response that may elicit help from other individuals

Chapman et al. (1985) divide behavioural responses to pain into simple reflex and complex voluntary behaviours. Simple reflex measures include the tail-flick response in rats (Miczek et al. 1982), limb-withdrawal responses (Pippi & Lumb 1979; Moens et al. 2003) and head shaking in horses (Brunson & Majors 1987; Brunson et al. 1987). In general, the latency to response has been measured. Complex voluntary behaviours represent a purposeful act requiring supraspinal sensory processing i.e. pain perception is occurring. Molony & Kent (1997) classify responses as involuntary or voluntary,

where involuntary behaviours may be more complex than simple reflex responses, such as the extension of the hind limbs due to hyper-reflexia and increased muscle tone in post-castration lambs (Molony et al. 1993). A similar voluntary behaviour was described as the adoption of an immobile stance.

Reflex behavioural responses to pain may occur in human beings in states of anaesthesia and analgesia, where perception of the stimulus is not registered. Pain perception is indicated by voluntary actions (Kitchell 1987). Additionally, behavioural responses to pain may be classified as either spontaneous or evoked. Spontaneous behaviours represent general behavioural changes performed by the animal in response to a pain state. Evoked behaviour occurs in response to human interaction. Interaction tests include wound palpation, general approach, calling name and innocuous touch.

1.6.1 Spontaneous Behaviour

1.6.1.1 Facial Expression

Facial expression has frequently been used as a tool for the identification and assessment of pain in human adults (Craig & Patrick 1984; Hill & Craig 2002), the elderly (Hadjistavropoulos et al. 2002) and neonates (Johnston & Strada 1986; Grunau & Craig 1987; Grunau et al. 1990; Craig et al. 1993; Lindh et al. 1997). Parameters such as brow bulge and eye squeeze are included in the facial action coding system (FACS) and neonatal facial coding system (NFCS), producing reliable and repeatable results (Craig et al. 1993). Although cited as key to the identification of pain in animals, characteristics of facial expression have yet to be objectively assessed in animals (Short 1999). However, a number of anecdotal sources have described a facial expression of concern or uneasiness (Fraser 1969; Casey 1999) as a general indicator of pain in the horse. Other descriptions of non-specific indicators of severe pain include a wild, distracted appearance and a fixed stare and eye rolling (Fraser 1969; Casey 1999). More objective assessment of ear position has found a decreased time spent with ears forward following arthroscopy (Price et al. 2003). Objective characterisation of facial expression in animals may be difficult due to less obvious movement/changes in expression. Additionally, the characterisation of changes in facial expression in humans is based

mainly around the analysis of photographic or video recordings; however, the 3D facial shape of most non-human animals would add complications to analysis.

1.6.1.2 Posture and Guarding behaviour

Posture often changes as the animal adopts position which results in minimal pain. Guarding behaviour reduces stimulation of the affected area through pressure or movement; a classic example being the reluctance to bear weight seen in chickens following sodium urate induced arthritis (Gentle et al. 1999; Hocking et al. 2001; Hocking et al. 2005).

Changes in standing position may result in abnormal postures or states. For example, statue standing occurs in castrated calves (Molony et al. 1995) and lambs (Molony et al. 1993) and following mulesing in sheep (Fell & Shutt 1989; Grant 2004) represents an abnormal state characterised by a normal posture which is performed without other movement for long periods of time. In castrated lambs abnormal postures standing postures have been recorded where the animals stand with the legs splayed (Graham et al. 1997; Kent et al. 1998; Archer et al. 2004). Laminitis results in a characteristic stance with the legs positioned underneath the stomach, weight born on the heels and shifted from fore to hind feet (Budras et al. 2001; Hood et al. 2001; Rietmann et al. 2004). In addition to the change of posture, horses may repeatedly lift alternate forefeet (Obel 1948; Rietmann et al. 2004). These behavioural changes reduce pressure on the toe area and forefeet, which are predominately affected. Leg lifting may be seen in other limb and foot pains, along with a reluctance to move and 'board-like' posture (Taylor et al. 2002b).

Changes in recumbent posture, generally an increase in lateral recumbency, are related to acute musculoskeletal and general chronic pain states in the horse (Matthews 1992). Similarly, following ovariohysterectomy, bitches spent more time sleeping in lateral recumbency than controls (Firth & Haldane 1999). In abdominal pain, horses may lie in dorsal recumbency with limbs in the air (Fraser 1969). Abnormal lying is a characteristic feature of castration pain in lambs, with animals adopting a ventral position with legs stretch out to the side (Graham et al. 1997; Kent et al. 1998; Archer et

al. 2004). Alteration in recumbent posture are also seen in cats, which appear 'sprawled on the floor' with affected limb raised and extended, following sodium urate arthritis (Okuda et al. 1984).

Head position has been identified as an indicator of post-surgical pain, with an increase in lowered head position in arthroscopy patients (Price et al. 2003); a posture generally associated with depression (McDonnell 2003). Similarly, post-mulesing behaviour in sheep includes increased time spent with head down (Fell & Shutt 1989; Grant 2004).

1.6.1.3 Locomotive behaviour

Locomotive behaviour may be increased or decreased depending on the type, location and severity of pain. Reduced locomotion is associated with laminitis (Budras et al. 2001) and other painful conditions of the limb and foot and may result in inability to obtain food and water and escape from predators or conspecifics. Abnormal forms of locomotion may also be seen. For example, backward locomotion was increased following caustic dehorning of calves (Graf & Senn 1999). Changes in gait to reduce pressure on affected limbs are also associated with limb and digital pain; however, these have been discussed in section 1.5.2.2.

Increased resting or reduced activity may reflect pain-induced immobility and/or conservation of energy for recuperation. Post-castration piglets show increased sitting and standing-inactive, but less lying. This suggests that the type resting behaviour may be altered dependant on the most comfortable position for the animal (see posture/guarding behaviour) (Taylor et al. 2001). Similarly, increased resting is a characteristic feature of sodium urate induced arthritis in the chicken (Gentle et al. 1999; Hocking et al. 2001; Hocking et al. 2005). In horses, increased resting behaviour has been seen following exploratory celiotomy (Pritchett et al. 2003) and may also be associated with the decreased exploratory behaviour reported following arthroscopic surgery (Price et al. 2003).

Restlessness in the horse, often displayed as increased getting up and lying down, has been associated with both abdominal, colic or sharp sudden pains (Lowe 1992; Taylor et

al. 2002b). Calves also show increased restlessness following castration (Molony et al. 1995).

1.6.1.4 Feeding behaviour

Pain related anorexia is well recognised in association with chronic and acute pains, often leading to weight loss and reduced growth. Pain may indirectly result in reduced feed intake due to lack of mobility (Short 1999). Pain-associated loss of appetite has been noted in rodents (Wright et al. 1985; Morton & Griffiths 1985) and used as an indicator variable for the comparison of different analgesic regimens (Flecknell & Liles 1991; Liles & Flecknell 1992). Decreased time spent grazing was observed in sheep following mulesing (Fell & Shutt 1989) and less suckling, licking and chewing was seen in castrated piglets than control piglets (Hay et al. 2003). Horses may show decreased eating and drinking during acute musculoskeletal pain (Matthews 1992). Snatching at food without really eating has been reported in cases of severe pain (Taylor et al. 2002b). Dental pain may result in abnormal chewing, including reductions in speed and range of movement and the presence of slurping noises. 'Quidding' may also be seen in these horses, where semi-masticated food falls from the mouth (Lane 1994; Dixon & Dacre 2005). Care should be taken when examining feeding behaviour as in many post-surgical pain states animals will be highly motivated to feed due to pre-operative starvation.

1.6.1.5 Aggression

An aggressive or defensive response to touch may occur when an animal is in pain (see evoked behaviour) (Short 1999). As flight animals, horses will instinctively avoid aggressive encounters. However, when once confined they may respond aggressively to threat or pain (Casey 1999). Generally, horses in pain become more difficult and fractious during handling (Ashley et al. 2005). Lack of cooperation with handlers has been observed in Przewalski horses suffering from laminitis (Budras et al. 2001). Observation of an aggressive response to palpation or interaction (see evoked behaviour) may be a good indication of pain; however, other factors such as previous experience and handling may significantly affect the response.

1.6.1.6 Vocalisation

Assessment of vocalisation in response to painful procedures has been used to examine tail docking/castration in pigs (Noonan et al. 1994; White et al. 1995; Weary et al. 1998) and branding in cattle (Watts & Stookey 1999). Analysis of neonatal pain cry is also (Grunau & Craig 1987; Grunau et al. 1990) a useful indicator of pain in non-verbal humans. Frequency and duration of vocalisations can be recorded, along with a variety of acoustic parameters. Frequency of different *types* of vocalisation may also be used (Noonan et al. 1996). Spectral analysis of sound recordings enables the examination of differences in pitch and sound intensity (Watts & Stookey 1999).

Whilst vocalisations may be used as indicators of pain in species such as pigs (White et al. 1995), it should be remembered that calls may also reflect fear and anxiety as well as pain (Conzemius et al. 1997). Horses display a number of different vocalisations (McDonnell 2003), although their natural history suggests they are unlikely to use vocal communication to elicit help from others in their groups. Groaning and grunting have been anecdotally associated with abdominal pain (Matthews 1992; Casey 1999) and snorting with head pain (Taylor et al. 2002b).

1.6.1.7 Social Behaviour

Social behaviour is an important indicator of animal pain, with isolation often occurring as an early sign of pain (Anil et al. 2002). Isolation may be protective, reducing possibly painful interactions with conspecifics (Mellor et al. 2000). Following mulesing, sheep reduced social interaction and appeared withdrawn from the flock (Fell & Shutt 1989). Piglets also spent more time isolated from littermates immediately following castration (Hay et al. 2003) and additionally showed behavioural desynchronisation with littermates. The majority of pain studies in the horse have been performed on individually housed animals, limiting the understanding of the effects of pain on social behaviour. However, anecdotally chronic pain has been cited to result in modifications to normal social behaviour (Matthews 1992).

1.6.1.8 Absence of Normal Behaviour

The absence of normal behaviour may be one of the most striking signs of pain in animals. A good understanding of species specific behaviour is therefore of great importance (Anil et al. 2002), as well as an understanding of the individual animal. The reduction of non-essential maintenance behaviours such as grooming, have been reported post-operatively in horses (Eager 2002) and dogs (Firth & Haldane 1999). This may result from a need to conserve energy or an unwillingness to move. Position in the stable has been found to alter in acute post-operative pain (arthroscopy), with animals spending more time towards the back of the stable (Price et al. 2003). This change in behaviour may reflect withdrawal and general lack of interest in their environment. Anecdotal reports also suggest that horses with abdominal pain will back themselves into a corner (Fraser 1969).

1.6.1.9 Abnormal Behaviour

A huge variety of behaviours have been considered to be associated with pain in the horse. A behaviour may be considered abnormal if it:

a) does not occur in the normal behavioural repertoire of the horse i.e. it is a novel behaviour
b) is occurring out of context (i.e. a displacement behaviour)
c) is occurring at an abnormal frequency

Novel behaviours associated with pain are most commonly seen in colic, when the horse is experiencing a specific and severe pain. Table 1.1 lists a number of novel behaviours reported to occur in equine pain.

Behaviour	Location/type of pain
Kicking/biting abdomen	
Looking at flanks	Acute abdominal
Thrashing	
Throwing self on floor	
Tooth grinding	Acute abdominal / back pain
Head pressing	Head pain
Head tilting	Neck/back pain
Stumbling	Back pain
Straining	Acute abdominal
Nostrils dilated	General
(Fraser 1969; Jöchle et al. 1989; Lowe 1992; Matthews 1992; Taylor et al. 2002b)	

Table 1.1 Abnormal behaviour performed in association with pain in the horse.

Displacement behaviours are those which occur out of context. A text book example is sleeping behaviour during a fight in oystercatchers (McFarland 1999). Licking and chewing, as seen in post-surgical pain (Price et al. 2003), often occurs in anticipation of food. However, this behaviour may also be considered a displacement behaviour in situations where food is provided and the behaviour occurs completely out of context. Care must be taken in the interpretation of this type of behaviour. Abnormal sweating and muscle tremors may be general signs of pain in the horse (Fraser 1969), although may not be reliable markers of moderate or mild pain. Tail flicking is considered a general indication of discomfort in horses (Taylor et al. 2002b) and has been reported in post-castration piglets (Hay et al. 2003) and following dehorning in calves (Graf & Senn 1999; Vickers et al. 2005). However, fly activity must also be assessed as this may both affect the behaviour of the animals and increase where wounds are present. Problems such as difficulty in bitting and head shaking whist riding may be a sign of dental pain (Lane 1994); bolting, bucking and rearing may be indicative of back pain (Taylor et al. 2002b). Horses may often perform these behaviours when frightened. However, when they occur out of context, pain may be a contributory factor.

Increased rolling in response to acute abdominal pain is possibly the most commonly considered abnormal pain behaviour in the horse (Fraser 1969; Matthews 1992; Taylor et al. 2002b). Increases in pawing and stretching are also seen in equine colic (Fraser 1969; Lowe 1992). Similar behaviours such as stamping and easing quarters are indicative of castration pain in calves (Molony et al. 1995) and lambs (Graham et al. 1997; Kent et al. 1998; Archer et al. 2004). Head shaking is often performed by horses with back or head pain (Taylor et al. 2002b). These are normal behaviours which are considered abnormal due to the increased or decreased frequency of their performance.

1.6.1.10 Stereotypical Behaviour

Stereotypical behaviour may also be considered a form of abnormal behaviour. A stereotypy can be defined as a '*behaviour pattern that is repetitive, invariant and has no obvious goal or function*' (Mason 1991). In horses common stereotypical behaviours include weaving, crib-biting and wind-sucking (McDonnell 2003). Stereotypical head

'bobbing' has also been cited in association with acute musculoskeletal pain (Matthews 1992).

1.6.2 Evoked Behaviour

Evoked behaviour examines responses to external stimuli, such as innocuous touch, and responses to people and noise in general. In situations where pain is mild and spontaneous behaviour unchanged, examination of evoked behaviour may be a more sensitive tool for assessment, inducing a more noticeable response (Rutherford 2002).

Response to wound palpation has been used as a tool for pain assessment in dogs (Reid & Nolan 1991; Nolan & Reid 1993; Lascelles et al. 1997), cats (Bley et al. 2004), cows (Chevalier et al. 2004) and sheep (Thornton & Waterman-Pearson 1999). Typical responses include twitching, struggling, kicking, jerking, vocalisation, flinching and stepping away (Chevalier et al. 2004).

Response to human interaction has also been used, mainly for pain assessment in dogs (Lascelles et al. 1997; Holton et al. 1998; Firth & Haldane 1999). Standardised interaction tests included approaching cage, standing in front of cage, making noise at front of cage, and speaking to the dog (Holton et al. 1998; Firth & Haldane 1999). Responses to such tests in dogs have shown surgery dogs to remain in sternal recumbency during the interaction for longer than control dogs and perform less tail wagging and licking (Firth & Haldane 1999). When asked to generate a list of terms to describe pain in dogs, practising veterinarians included two evoked behavioural categories, response to touch and response to people (Holton et al. 2001). These categories included parameters such as aggressive, fearful and indifferent in response to people and flinching, growling and guarding in response to touch.

Palpation and interaction tests have been less frequently used in the assessment of equine pain. Houdeshell and Hennessey (1977) incorporated a palpation test into a multi-practice study assessing the efficacy of flunixin. Palpation responses were scored for pain, which decreased after administration. Preliminary research identified changes in evoked behaviour following equine castration, with castrate horses showing increased

time turned towards the handler and putting their ears back during an interaction test (Eager 2002). When interpreting responses to palpation or interaction tests in horses, it should be remembered that they are highly sensitive to touch (Casey 1999) and may react violently to innocuous stimuli if they are frightened (Taylor et al. 2002b). Clearly, this reaction cannot be attributed to pain.

1.6.3 Summary

Examination of pain behaviour in the horse is complicated by the inherent tendency for equines to mask behavioural signs of pain in stressful or frightening situations. In many clinical studies, horses are maintained in unfamiliar, hospital environments, isolated from their peers. This is likely to lead to apprehensive, nervous or excited animals (Taylor et al. 2002b). Domestication, socialisation, age, species, breed and environment may all affect behavioural responses to pain along with specific responses being attributed to the nature and location of pain (Conzemius et al. 1997; Casey 1999; Molony et al. 2002; Archer et al. 2004). Furthermore, the optimal indices for the assessment of a specific pain may vary with time (Molony et al. 2002). Despite these problems, behavioural assessment is perhaps the only practical technique for the assessment of pain in the clinical environment (Rutherford 2002). Behavioural responses to pain occur almost immediately and can give a good indication of the duration and phases of pain experience (Mellor & Stafford 2000). Additionally, observation of behaviour is non-invasive and so has no detrimental effects on the animal (Rutherford 2002). In order for the behavioural assessment of pain to be optimised, controlled and objective research is required to determine indices specific to a particular species, pain state, type and location.

1.7 PHYSIOLOGICAL ASSESSMENT OF PAIN

Pain induces a state of stress (Wolfle 2000), and it is the physiological changes associated with stress that can be measured to give indications of pain. Whilst these parameters are measured objectively, interpretation of changes identified is still subjective (Rutherford 2002). Physiological responses to pain-induced stress can be divided into those resulting from the activation of the hypothalamo-pituitary-

adrenocortical (HPA) axis and those resulting from the sympathetic-adrenomedullary (SA) axis.

1.7.1 The sympathetic-adrenomedullary Axis

The SA axis is concerned with the fast acting 'fight or flight' response, which enables the animal to act quickly to 'deal' with a stressful situation. SA activation increases blood flow to the muscles, releases energy into the blood stream and diverts blood from non-essential systems such as the digestive system. The secretion of adrenaline and noradrenaline into the blood stream results in increased heart rate, respiration rate and body temperature as well as changes in blood pressure and pupil dilation. A number of studies have directly examined circulating adrenaline and noradrenaline, whereas others have focussed on the more easily observable responses.

1.7.1.1 Adrenaline and Noradrenaline

The identification of increased plasma catecholamines (adrenaline and noradrenaline) concentrations in association with animal pain has shown some inconsistency. One study examining responses to freeze and hot-iron branding in cattle found no catecholamine response (Lay et al. 1992a) whereas two other studies found a significant increase in response to the same stimuli (Lay et al. 1992b; Lay et al. 1992c). These differences may be attributable to different breed characteristics. Urinary catecholamine was measured in piglets but showed no significant alterations following castration (Hay et al. 2003). Examining plasma catecholamine in laminitic horses, Rietmann et al. (2004) found no change in levels following administration of analgesic agents.

1.7.1.2 Heart Rate

Anecdotally, heart rate has been considered a general sign of pain in horses (Fraser 1969) and has also been cited by U.K. veterinarians as a primary parameter for pain assessment (Price et al. 2002). Experimental evidence for a pain-associated elevation of heart rate in horses is inconclusive. Following exploratory celiotomy, heart rate was elevated in surgical, but not control (having undergone general anaesthesia) horses (Pritchett et al. 2003). These effects may, however have been related to colic-associated shock and endotoxemia rather than pain (Taylor et al. 2002b). Heart rate was not altered

post-operatively in arthroscopy patients (Price et al. 2003) and could not be used to distinguish between those patients given analgesia and placebo (Raekallio et al. 1997a). Analgesic treatment of chronic pain in association with laminitis resulted in a decrease in heart rate, suggesting previous elevation in association with pain (Rietmann et al. 2004).

Research in other species is similarly inconclusive. White et al. (1995) found a significant rise in heart rate for 2-3 minutes following castration in piglets. However, no reduction in this rise could be shown in response to analgesia. A similar lack of response to analgesia has been reported in cattle (Chevalier et al. 2004) and dogs (Firth & Haldane 1999). Results of the examination of heart rate responses to feather removal in chickens were variable and there was no correlation between heart rate response and pain behaviour (Gentle & Hunter 1990).

Taylor et al. (2002b) suggested the unidentified alterations in heart rate in clinical studies to be due to insufficient painful stimuli and the presence of cardiovascular modifying drugs. Heart rate may also be affected by a large number of interfering variables such as eating, exercise and extraneous events (Molony & Kent, 1997). Furthermore, evidence suggests that in cattle, heart rate responses may be significantly altered by temperament (Lay et al. 1992b). These factors in combination imply that heart rate may not be an adequate indicator of clinical pain, in cases where horses may be undergoing drug therapy, stressful procedures and may be housed in a completely novel environment.

1.7.1.3 Respiration Rate

As with heart rate, increased respiration rate is frequently cited as a general indicator of pain (Fraser 1969). Research in other species suggests respiration rate to be a poor indicator of pain in cats (Cambridge et al. 2000), dogs (Firth & Haldane 1999) and cattle (Chevalier et al. 2004). Similarly, clinical equine research has found no difference in respiration rate in post-operative arthroscopy (Price et al. 2003), castration (Eager 2002) or exploratory celiotomy (Pritchett et al. 2003) patients compared to clinically normal controls.

1.7.1.4 Body Temperature

Very little scientific information is available on the effect of pain on body temperature in the horse. In other species, body temperature has been shown to be a poor indicator of pain in cattle and dogs (Firth & Haldane 1999; Chevalier et al. 2004).

1.7.1.5 Blood Pressure

Increased blood pressure was identified following feather removal in chickens, but, there was no correlation between blood pressure and behavioural parameters (Gentle & Hunter 1990). Blood pressure has been described as weak discriminator of painful and pain-free animals, following ovariohysterectomy in bitches (Firth & Haldane 1999). As with body temperature, there is no scientific evidence to highlight blood pressure as a potential indicator of pain in the horse.

1.7.2 The Hypothalamo-pituitary-adrenocortical Axis

The HPA axis coordinates slow, long-lasting metabolic and anti-inflammatory responses, mediated by corticosteroid hormones.

1.7.2.1 Cortisol

The function of cortisol as a stress hormone means that it is released in a variety of stressful situations (Taylor et al. 2002b) and therefore cannot be considered as a direct indicator of pain. However, measurement may provide an integrated measure of pain and non-painful stressors associated with a procedure (Jongman et al. 2000). Other factors such as high individual variation, diurnal variation and effects of sampling procedures are also of concern (Molony & Kent 1997). Cortisol is most commonly measure in the blood plasma, but urine, saliva and faeces may also be used. Whilst plasma or saliva concentration is most easily measured (Molony & Kent, 1997), concerns over the effect of sampling stress (Merl et al. 2003) and anticipatory reactions (Beerda et al. 1996) have prompted the exploration of other techniques. Examination of urinary and plasma cortisol in dogs found little correlation (Beerda et al. 1996). However, during the experimental emptying of the bladder a significant correlation was found (Jones et al. 1990). These results highlight a significant problem with lag time and sample accuracy using non-invasive faecal or urinary sampling. Further

investigation of the delay between onset of stressor and urinary cortisol response may improve the accuracy of this technique (Beerda et al. 1996).

Plasma cortisol responses have been used in a number of studies based on the identification of 'best practice' for castration and tail docking of lambs (Kent et al. 1993; Graham et al. 1997; Kent et al. 1998). However, differences in age and breed of lambs resulted in differences in baseline values. In order to compare cortisol responses to a variety of techniques for castration and tail docking in different breeds and ages, the integrated cortisol response analysis system has been developed (Mellor & Stafford 2000). The integrated measure examines the area between the plasma cortisol concentration-time curve and a horizontal line drawn through the pre-treatment value (Mellor & Murray 1989b).

Secretion of cortisol is known to reach a 'ceiling' point of maximum secretion (Rutherford 2002). This ceiling effect may interfere with value as a index of pain severity (Molony et al. 2002). The inclusion of an adrenocorticotropic hormone (ACTH) challenge group into pain studies (Mellor & Murray 1989b; Lester et al. 1991) can be used to determine if cortisol 'ceiling' had been reached.

In clinically normal animals, plasma cortisol concentration will fluctuate diurnally. A significant diurnal rhythm has been identified in the horse with peak values in the morning and a trough in the early evening (Bottoms et al. 1972; Irvine & Alexander 1994). Obliteration of these rhythms can occur with environmental change, such as maintenance in a barn or yard (Irvine & Alexander 1994). However, if animals are fully accustomed to artificial housing, cortisol rhythms are re-established.

Clinical pain studies examining cortisol levels in horses have proved inconclusive. Increased plasma cortisol, in comparison with normal controls, is reported for horses with colic (Hodson et al. 1986; Merl et al. 2003), although variation in concentrations was not correlated with severity (Merl et al. 2003). Following exploratory celiotomy, cortisol was higher than with general anaesthesia alone (Pritchett et al. 2003). These animals were maintained in artificial conditions, transported, and/or introduced in to a

novel, hospital environment. These environmental changes may have had a significant effect on cortisol levels. Examining plasma cortisol following arthroscopic surgery compared to a single pre-operative value, (Raekallio et al. 1997a; Raekallio et al. 1997b) found no significant effect. Following surgery, horses administered phenylbutazone had significantly lower plasma cortisol levels than those given placebo. This suggests that animals may have been in pain prior to surgery, when pre-operative samples were taken, and in less pain following the administration of analgesia. Whilst the authors suggest that a lack of a pre-, post-operative difference may be due to diurnal variation, it is not stated whether or not animals were given enough time to habituate to their new surroundings. Stress of transportation, new housing and social isolation could all cause alterations in plasma cortisol. Increased cortisol concentrations have been reported for up to two days following equine castration (Merl et al. 2003). However, the procedure was performed under general anaesthesia, and the study lacked a drug control. It is, therefore, impossible to distinguish between the effects of pain and of the procedure. In a single study examining cortisol in association with chronic pain (laminitis), Reitmann et al. (2004) found no alteration in levels following administration of analgesia.

Cortisol may show increases in association with pain in horses. However, as a large variety of factors can affect cortisol release, its effective use in a clinical setting is limited. Taking blood samples from clinical cases that are not sufficiently habituated to the procedure may affect results. Faecal sampling may only be useful for retrospective examination (Merl et al. 2003) and therefore not clinically viable. In a tightly controlled experimental situation, cortisol assays may be of use in the validation of other, more specific biomarkers of pain, although the relevance of such experiments to clinical pain assessment is debatable.

1.7.3 β-Endorphin

A number of studies have investigated the use of β-endorphin as an indicator of post-operative pain in horses. Increased β-endorphin levels have been reported following application of a twitch and naso-gastric tubing (Hydbring et al. 1996). However, it is not

yet determined if this is procedure-specific or a response to pain. Clinically, β-endorphin has been shown to rise in association with colic, but this may be attributable to shock (McCarthy et al. 1993). Similarly, post-operative increases were identified following arthroscopy, although the measure was not sensitive enough to discriminate between those animals given placebo and phenylbutazone analgesia (Raekallio et al. 1997a). No change was present in chronic lameness (McCarthy et al. 1993). As a retrospective measure, β-endorphin data may be of use in the monitoring of stressful situations, but not as an immediate indicator of pain. Additional problems include large individual variation in the equine population (Raekallio et al. 1997a) and the analgesic properties of β-endorphin, which may indicate that the animal is actually coping with the pain (Taylor et al. 2002b).

1.7.4 Summary

The examination of physiological variables is often considered 'more objective' than behavioural indices. Whilst being easily measured, these indices are not pain specific and may be significantly altered by factors such as exercise, feeding, sexual excitement and environmental stress. Modification of 'normal' values may also occur in association with disease and not pain *per se*. Special consideration of the effects of pharmacological therapy on physiological systems, especially cardiovascular effects, is of particular importance, as few studies address either this issue or the possible effect of highly significant inter-species differences. These fundamental limitations mean that, whilst the comparison between treatment and control groups may identify differences in physiological indices, monitoring of individual animals may be of limited use (Rutherford 2002).

1.8 EXPERIMENTAL DESIGN IN ANIMAL PAIN ASSESSMENT

Whether examining behavioural and/or physiological indicators of pain, a number of experimental designs have been employed in the study of animal pain. Pain may be experimentally induced (section 1.9) or arise from spontaneous disease or clinical surgical procedures (section 1.10). Studies may compare between 'pain-free' and 'painful' animals, those given different analgesic agents or placebos or make 'before'

and 'after' comparisons monitoring the same individual peri-operatively for instance. The use of analgesic agents as a tool for the identification of pain-related behavioural and physiological parameters and studies in which animals are given the opportunity of 'self-administration' of analgesic agents are discussed below.

1.8.1 Response to exogenously administered analgesic agents

Beneficial effects of analgesic agents provide strong evidence of previous pain (Sanford 1992). Monitoring responses to exogenously administered analgesic agents is also a commonly used technique for the identification of potential indicators of animal pain, particularly in the identification of '*best practice*' for common agricultural husbandry techniques (Dinniss et al. 1999; Thornton & Waterman-Pearson 1999). In equine practice, analgesia is a diagnostic tool frequently used in the assessment of lameness and back pain. For example, blocking with local anaesthetic is a common tool in the investigation of lameness.

Whilst this approach may provide crucial validation of previously identified parameters, it has been construed as a circular argument, in which behaviour is used to assess analgesic efficacy and analgesic agents used to assess the potential of behaviour as pain indicators (Flecknell 2000). Furthermore, species may respond in very different ways to a specific analgesic agent (Benson & Thurmon 1987), with drugs effective in human medicine being inadequate in other animals (Wall 1992). Furthermore, intra-species responses may vary with age, sex and breed (Molony & Kent 1997). Alterations in behaviour following analgesia may be drug but not pain related, with some agents resulting in depression of the central nervous system (Taylor et al. 2002b). Provision of a 'pain-free' control group may help identify drug related changes, the effects of which could then be incorporated into a pain assessment protocol.

1.8.2 Self-administration of analgesic agents

Clinical studies in human beings suggest that patient-controlled administration is a valid and reliable indicator of chronic pain (Portenoy & Hagen 1990). In terms of animal pain, it is suggested that if uptake of an analgesic agent reduces pain, it will reinforce further uptake (Kupers & Gybels 1995). The concept of self-administration (SA) as a

tool for the identification of animal pain was originally introduced by Colpaert et al. (1980), who found that rats with experimentally induced-arthritis consumed more analgesic agent (opioid) than normal animals. The validity of these results was further examined by Colpaert et al. (2001), determining that the conflicting effects of opioid dependence and intrinsically rewarding nature of self-administration were not occurring and that self-administration was in fact mediated by the presence of pain. The authors therefore concluded SA to be a useful tool for the identification of chronic pain in animals (Colpaert et al. 2001) which may demonstrate hyperalgesia but not persistent pain. Whilst the nociceptive chronic pain produced by arthritis produced SA, given the opportunity rats experiencing neuropathic pain did not self-administer opioids (Kupers & Gybels 1995). Similarly, Danbury et al. (2000) found lame broiler chickens to consume a greater amount of drugged feed than sounds birds, and that this increase could be titrated against severity of lameness. As far as the author is aware, there are no studies examining self-administration of analgesic agents in the horse.

1.9 EXPERIMENTAL PAIN STUDIES IN THE HORSE

Experimental pain models are most frequently used for the assessment of analgesia and the study of nociceptive mechanisms. Many experimental pain states are generated in laboratory animals through the injection of pain-producing chemical substances such as formalin (Coderre et al. 1993; Abbott et al. 1995; Abbott et al. 1999), carrageenan, complete Freund's adjuvant, capsaicin (Dubner & Ren 1999) and sodium urate (Okuda et al. 1984; Gentle 1997; Hocking et al. 2005). However, the use of these substances has not been reported in the horse: the majority of experimental-generated pain states are produced using the methods described below.

1.9.1 Dental Dolorimetry

Dental dolorimetry examines the response to stimulation of the tooth pulp nerves through the implantation of an electrode into the dentine layer of a canine tooth (Brunson & Majors 1987; Brunson et al. 1987). A head lifting withdrawal or a jaw-opening response was considered a strong positive indicator, whereas a weak-positive response was characterised by twitching of the neck muscles.

1.9.2 Balloon Model

Inflation of a balloon inside the rectum or caecum has been used as a model of visceral pain in an number of studies assessing analgesic efficacy (Pippi et al. 1979; Kalpravidh et al. 1984a; Kalpravidh et al. 1984b). The technique can be used to exert a constant pressure of intermittent periods of pressure by varied rates of balloon inflation (Lowe 1992). Generally, reactions such as kicking are examined by frequency of performance - a methodology with no clear end point (Kamerling et al. 1989). Attempting objectification, reactions have been sensed by a accelerometer fitted on the animal (Pippi et al. 1979; Kalpravidh et al. 1984b) and end points are determined by movements strong enough to obtain a specific reading. Clinical relevance of the model has been questioned as, using pressure, the balloon model mimics flatulent colic, whereas most colic includes an element of inflammatory pain (Kamerling et al. 1989).

1.9.3 Electrical stimulation

To study 'deep pain' Stenberg et al. (1986) implanted stimulating electrodes into the chorium of the hoof. Reactions to pain included shoulder muscle contraction, lifting hoof, blinking, lifting head and pricking ears. Shoulder movement was considered the most sensitive measure.

1.9.4 Summary

Analgesic effect may depend on manner of testing. For example, Moens et al. (2003) found differences in peak thresholds using mechanical and electrical stimulation for the assessment and comparison of xylazine, romifidine and detomidine. This may be due to differences in the nociceptive pathways.The use of experimental pain models may be vital for the study of pain mechanisms and assessment of analgesic agents. However, this work does little to increase our knowledge of the assessment and management of clinical pain, which may be continuous and vary in type and severity. Effective analgesic doses in this type of study may be very different from those required in a clinical setting (Taylor & Houlton 1984; Flecknell 1994).

1.10 CLINICAL PAIN STUDIES IN THE HORSE

The study of equine clinical pain has received comparatively little attention, with the majority of studies primarily focusing on the assessment of analgesic agents rather than the identification of parameters for the assessment of pain. A number of studies have utilised a variety of clinical situations, including elective (fracture fixation, arthroscopy and rig castration) surgery (Johnson et al. 1993); arthroscopy (Raekallio et al. 1997a) and colic (Jöchle et al. 1989) for the assessment of analgesic drugs. Concentrating on the identification of key parameters in the assessment of post-operative pain, authors have used a variety of models including arthroscopy (Price et al. 2003), exploratory celiotomy for colic (Pritchett et al. 2003); orthopaedic surgery (Raekallio et al. 1997b) and castration (Eager 2002). However, due to a number of experimental constraints, the validity of these studies may be questioned. The quality of control groups has varied throughout the studies, for example the complete lack of control animals in one study (Raekallio et al. 1997b). Whilst some authors attempted to control for experimental procedure (Eager 2002; Price et al. 2003) with pain-free animals housed in similar conditions, the effect of anaesthetic and analgesic drugs given were not controlled for in these studies. Additionally, where control animals are not subjected to anaesthetic and analgesic drug regimes they may also not experience similar periods of food deprivation (Price et al. 2003). Feeding motivation following starvation may be high and produce a significant effect on behaviour, which, without feed-deprived control groups, cannot be distinguished from changes due to pain. Pritchett et al. (2003) utilised animals presented for magnetic resonance imaging as a general anaesthesia without surgical insult control group. However, these animals exhibited some degree of lameness and so cannot be considered as 'clinically normal' and 'pain-free' which may affect results obtained. The use of animals as 'their own controls', with baseline data taken pre-operatively, may control for individual variation in variables such as experience and age. However, the use of such data may be limited in orthopaedic or arthroscopy patients where animals are likely to be experiencing some level of pain prior to surgery (Raekallio et al. 1997b; Price et al. 2003). Where castration is used as a model, these data may be more valid as animals are generally pain-free pre-operatively (Eager 2002).

Experimental variation has been increased in some circumstances by unstandardised drug protocols in surgery animals (Raekallio et al. 1997b; Eager 2002; Price et al. 2003), possible variation in presenting conditions (Raekallio et al. 1997b; Price et al. 2003; Pritchett et al. 2003) and the use of solely subjective techniques for the assessment of behaviour (Raekallio et al. 1997b).

Raekallio et al. (1997b) aimed to improve on the use of subjective scoring systems through objective assessment of behaviour and scoring bearing of weight and head position with a simple descriptive scale. 'Overall' subjective pain scores were also used. Behavioural observations of 1 minute duration were taken using time sampling and then a general score given to head position, bearing of weight and subjective pain score. These scores were then all summed to form a composite score. Slight differences were seen between behaviour in placebo and phenylbutazone animals but these were not significant. Whilst the study aimed to assess the efficacy of post-operative phenylbutazone in comparison with placebo, no control for the possible behavioural effects of phenylbutazone was present. Additionally, the study examined very few observation points and for limited time periods which may have affected the accuracy of behavioural sampling.

In a wide ranging trial Johnson et al. (1993) utilised a number of clinical conditions such as fracture fixation, arthroscopy and rig castration to assess the efficacy of three NSAID's. Postoperative pain was scored using a pain score of 0 = severe discomfort to 4 = no discomfort. It was difficult to assess results because 'abnormal' behaviour was but not characterized in the paper. Similarly, when examining the effects of butorphanol, flunixin and xylazine on clinical colic Jöchle et al. (1989) created a composite score from a range of behaviours (sweating, kicking, pawing etc). If behaviours used had been previously validated, then the system may have been more practically effective. However, the use of a composite score meant that it was not possible to identify which behaviours changed and which remained the same.

1.10.1 Summary

The use of experimental models of pain has advanced the knowledge of pain neurophysiology greatly and is crucial for the development of novel analgesic strategies. The ability to precisely control stimuli and environment reduces experimental variability. However, it may be difficult to relate experimentally induced pain to clinical conditions, where pain may vary in type, duration, phase and location (Chapman et al. 1985). The use of clinical conditions for the investigation of tools for the assessment of pain in animals may therefore be of particular importance. The environment and situation in which many of these studies are conducted may cause stress, thus modifying behaviour. Habituation to such environments is crucial to the integrity of such studies, enabling discrimination between stress and pain behaviour. Additionally, animals of a particular species, arriving at a veterinary clinic will be from diverse genetic and environmental backgrounds, unlike genetically engineered laboratory strains. Whilst this may increase experimental variation greatly, it is indicative of the 'normal' population and so relevant to the challenges faced in clinical practice.

1.11 BASIS FOR AND AIMS OF THE EXPERIMENT

Research into the assessment of equine clinical pain is in its infancy, with the majority of citations referring to subjective observations and clinical experience. Whilst this anecdotal evidence (especially where experienced clinicians and carers are concerned) is highly valuable, controlled objective research is required to accurately determine most effective indices for the assessment of equine pain, in general and in response to specific conditions. Previous studies have provided a substantial platform for future study and research utilising optimal pain models; standardised drug protocols and objective assessment techniques are needed to develop a sensitive, reliable and reproducible technique for assessment of clinical pain in horses. The aim of these studies is to characterise behavioural pain responses in horses, identifying general and specific behaviour of acute post-surgical and chronic pain states. A comprehensive behavioural ethogram was developed and a wide variety of behaviours observed, in order to identify potential biomarkers of pain, to assess the validity and sensitivity of indices and to eliminate redundant indices as recommended by Molony and Kent (1997).

The development and validation of objective multi-dimensional pain assessment protocols for general and specific conditions will have multiple benefits for equine welfare. An assessment system will not only help those experienced in pain assessment to continually monitor an animal, but will be of great use to the less experienced clinician or horse owner for the identification and assessment of pain in their animals. The ability to accurately assess pain severity will minimise the number of animals suffering due to lack of pain relief but also reduce unnecessary and inaccurate dosing. An accurate pain assessment protocol will also be of use within the scientific community and can be employed in the evaluation of analgesic efficacy and pain management protocols. It will therefore aid the identification of '*best practice*' in a variety of procedures and treatments. Assessment systems can be used to determine relative severity and duration of pain in common procedures, enabling the development of optimal pain management protocols.

The overall aim of these experiments is to objectively identify behavioural biomarkers of both acute and chronic pain states in the horse. Further aims include;

1. Determination of the effects of sedative, anaesthetic and analgesic drugs on equine behaviour
2. Clarification of the effects of external factors such as diurnal variation and phases of pain.
3. Clarification of most important indices of assessment and elimination of redundant indices, to assist in the future generation of a succinct and efficient assessment protocol.

Examination of these aims generated the following null hypotheses to be tested;

1. There will be no significant difference in behaviour between 'painful' (castrate/laminitic) and 'pain-free' (control) horses.
2. There will be no effect of anaesthetic or sedative drugs on equine behaviour.
3. External factors such as time of day will have no influence on equine behaviour.
4. Behavioural variables measured will not discriminate between 'painful' (castrate/laminitic) and 'pain free' control horses.

CHAPTER TWO

GENERAL METHODOLOGY

2.0 INTRODUCTION

Testing the experimental hypotheses described in chapter one, required the examination of different experimental models; the implementation of a variety of techniques for the assessment of behaviour; and the use of a wide range of univariate and multivariate statistical analyses. As methodologies are similar between chapters, the following sections describe the experimental models and behavioural and statistical methodologies used to achieve the project aims and test the hypotheses.

2.1 EQUINE PAIN MODELS

The majority of equine pain studies have used experimentally generated pain states to assess the effectiveness of analgesic agents. These models have included the inflation of a balloon in the caecum or rectum (Pippi & Lumb 1979) and the implantation of tooth pulp electrodes (Pippi & Lumb 1979; Brunson et al. 1987). Determination of analgesic efficacy is gained from simple behavioural responses such as lifting of a leg or shaking the head. However, these models and responses may not applicable in a clinical setting where pain may be phasic, varying in duration and type (Taylor et al. 2002b).

Clinical trials have involved a number of common situations, including orthopaedic surgery (Johnson et al. 1993; Raekallio et al. 1997a; Raekallio et al. 1997b; Price et al. 2003), colic (Jöchle et al. 1989; Pritchett et al. 2003) and castration (Eager 2002). The validity of these studies may be affected by a number of experimental constraints including quality or indeed lack of control groups, unstandardised drug protocols and possible variation in presenting conditions. The sole use of unvalidated, subjective scoring techniques, where ratings may be based on anecdotal evidence, limits the use of results in the determination of actual responses to pain.

The studies reported in the following chapters aimed to perform a comprehensive assessment of equine pain behaviour through the examination of responses to both acute post-surgical and chronic pain states. Equine castration (under both standing surgical

sedation and general anaesthesia) was used as a model of acute post-surgical pain and laminitis as a model of chronic pain.

Pain management in an acute post-operative state is of particular concern as the effects of tissue damage and pain may be most severe and animals may not be in the best state to accommodate these effects (Crane 1987). Castration is the most commonly performed surgical procedure in the horse (Schumacher 1996), performed to minimise unwanted sexual behaviour and facilitate management (Houpt 1999). Castration is particularly useful as an experimental model as it allows the collection of baseline, pre-surgery data, enabling animals to act as their own controls. In addition to the identification of potential markers of acute pain, the study of responses to equine castration may aid the identification of 'best practice' for this procedure.

Management of chronic pain is crucial in the maintenance of welfare standards and minimisation of suffering in association with long-term injury and disease. Laminitis is a painful, debilitating condition, affecting approximately 7.1% of the U.K. equine population (Hinckley & Henderson 1996). The improvement of assessment for chronic painful conditions such as laminitis will help in the monitoring of disease progression, disease recognition and the identification of novel strategies for the management of severe, chronic pain

2.2 BEHAVIOURAL METHODOLOGY

Few studies of equine pain behaviour have involved the objective assessment of a variety of behavioural indicators through the measurement of frequency and duration of a wide range of states and events across and expanded time frame. Commonly, behaviours are included in a protocol for assessment without consideration of the specificity of each parameter, whether a particular range of behaviours show a linear representation of pain severity and whether a particular behaviour is of greater than another, for instance, both excessive activity and lethargy are considered signs of pain (Sawyer 1998).

The use of subjective methods of assessment can be biased if personal expectations influence judgement (Hansen 1997; Cambridge et al. 2000). Preconceived beliefs concerning pain severity associated with a specific condition or procedure may affect judgement (Holton et al. 1998) and interpretation of behaviour may be affected by philosophical differences between observers (Conzemius et al. 1997). The assumption that pain severity is directly correlated with tissue damage may be incorrect as pain tolerance may vary between individuals (Wolff et al. 1997), with breed (Archer et al. 2004), age and sex (Hansen 1997).

The current research aimed to objectively identify potential behavioural indicators of both acute and chronic pain states. The following sections describe the methodologies used to examine both spontaneous and evoked behaviour. Precise ethograms of normal and abnormal behaviour helped objectify observation and recording without personal bias and value judgment (Hansen 1997; Bath 1998).

2.2.1 Duration of Observation

The duration of observation for both castration models included 24 hours pre-intervention (surgery or anaesthesia) or baseline monitoring and 48 hours post-intervention monitoring. Laminitic and control horses were observed for a maximum period of five days. The establishment of duration of observation in both models was predominantly based on practical and ethical considerations. The maintenance of clinically normal, healthy control horses in a stable for 24 hours a day over long periods of time represents a considerable welfare concern and would not only be ethically unacceptable but would reduce the likelihood of owners volunteering their animals for the study. Following discussions with veterinarians and animal welfare scientists it was determined that a maximum of five days stabling would balance between the need to gain enough data without negatively impacting on animal welfare to a significant extent.

Determination of duration of observation for laminitic horses was therefore set at five days or until euthanasia was deemed necessary. The timeframes for castrations models, however, were complicated by a number of factors. Castration under general anaesthesia required horses to be admitted to the equine hospital. It was therefore

required that horses undergo a period of habituation to the hospital environment to minimise any behavioural change associated with response to the novel environment. A period of 12 hours was allocated for habituation, following which horses were monitored for 24 hours to gain baseline data. Prior to undergoing general anaesthesia, a period of starvation is required (12 hours). Baseline observation could not take place during this period of starvation as this could potentially effect behaviour and give an inaccurate pre-intervention assessment. The process of habituation, baseline data collection and starvation therefore took a minimum of 48 hours. Adding the time taken for surgery/anaesthesia and recovery to this meant that a period of 48 hours remained was considered realistic for the monitoring of post-intervention changes bearing in mind a maximum stabling duration of five days and allowing time for alterations in schedule etc.

2.2.2 Spontaneous Behaviour

Undisturbed spontaneous behaviour was remotely monitored using CCTV cameras (WV-BP100, Panasonic, Matsushita Electric Industrial Co Ltd., Osaka, Japan) and 24-hour time-lapse video recording equipment (AG-6124, Panasonic, Matsushita Electric Industrial Co Ltd., Osaka, Japan). Two or more cameras were used in order to obtain optimal view of the box. A quad box (RSB Real Time Quad Processor, R.O.C, Taiwan) allowed data from all cameras to be recorded on one tape and viewed at the same time. In castration under general anaesthesia and laminitis models, two cameras were positioned in the box, as shown in figure 2.1. In the castration under standing surgical sedation model, size and shape of lose boxes varied. Number and configuration of cameras was altered, therefore, to gain the best possible view of the box.

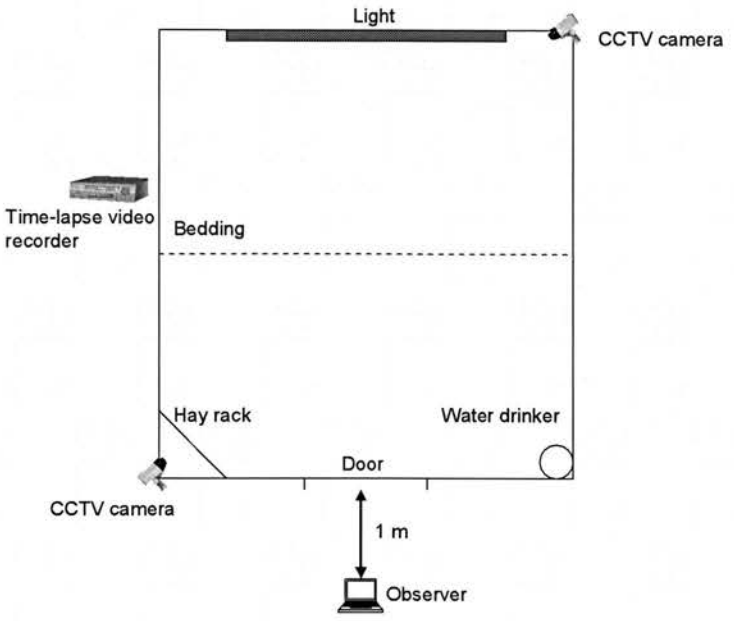


Figure 2.1 Camera configuration and layout of box in castration under general anaesthesia and laminitis models.

Detailed observations were required to detect subtle behaviours, such as lip tension and ear position, which may not be visible on CCTV recordings. These observations were performed by an observer positioned outside the stable, at least one metre from the door. Prior to commencing a recording the observer sat in position for at least 10 minutes to allow horses to habituate to their presence.

2.2.3 Evoked Behaviour

The examination of evoked behavioural responses may be more effective than spontaneous behaviour in species prone to pain masking and in mild pain states where other behaviours remain unchanged (Rutherford 2002). Evoked behavioural tests may include wound palpation and a variety of interactive tests such as response to approach, calling of name and reaction to noise. As detailed in section 1.6.2, palpation and interaction tests have predominately used in canines (Reid & Nolan 1991; Nolan & Reid 1993; Lascelles et al. 1997; Holton et al. 1998; Firth & Haldane 1999; Holton et al. 2001) but also in sheep (Fell & Shutt 1989; Thornton & Waterman-Pearson 1999), cats (Bley et al. 2004) and cows (Chevalier et al. 2004). However, evoked behaviour is rarely incorporated in equine studies. Houdeshell and Hennessey (1977) incorporated a palpation test into a multi-practice study assessing the efficacy of flunixin. Palpation

responses were scored for pain, which decreased after administration (Houdeshell & Hennessey 1977). Preliminary research identified changes in evoked behaviour following equine castration, with castrated horses showing increased time turned towards the handler and putting their ears back during an interaction test (Eager 2002).

When designing interactive or palpation tests for use in the horse, it should be remembered that horses are generally sensitive to touch (Casey 1999) and may react violently to innocuous touch if they are frightened (Taylor et al. 2002b). Responses to these types of tests should therefore be interpreted with care as a fear response is not indicative of pain. The interaction tests used in this thesis were designed to specifically relate to the type and location of pain. It was not considered possible, due to safety issues, to incorporate a test of wound palpation as has been used in other studies, of the acute post-surgical pain model used here. The test used for both castration models involved the handler approaching the horse and fitting a head collar. Standing on the near side, the handler then placed their hand half way down the horse's neck, and, applying a constant pressure, moving the hand slowly down the neck, across the shoulder and down to cup the girth. They then moved their hand under the belly towards the tail until they were approximately half way along the belly. If an extreme reaction occurred at any point, the test was terminated and analysis performed on the available information.

In the laminitic model, the handler entered the stable and fitted a head collar to the horse. If the horse was lying down and would not stand following three gentle pulls on the lead rope, the test was not performed. As before, starting on the near side, the handler placed their hand half way down the horse's neck and moved slowly down the neck and down the shoulder. Instead of moving their hand underneath the girth, the handler then ran their hand down the horse's forelimb until reaching the fetlock. The handler then removed their hand and moved to the horse's rear, placing their hand midway down the flank and ran their hand down the hindlimb, again until reaching the fetlock. This process was repeated on the off side.

2.2.4 Measuring Behaviour

Within this study, behaviour was recorded using *continuous* or 'all-occurrences' recording, to produce an accurate record of behaviour performed (Martin & Bateson 1993). *State* behaviours are behavioural patterns which have a relatively long duration for example, body postures. Duration of these behaviours has been recorded as proportion of the total sample duration. *Event* behaviours have a relatively short duration, for example, stamping or kicking. The frequency of these behaviours was measured (Martin & Bateson 1993).

Behavioural categories, such as oral or locomotive behaviour, contain a group of related *behavioural elements*. These elements include mutually exclusive state behaviours and associated event behaviours. As each behavioural element describes a single precisely defined behaviour, these have been termed *individual behaviours.*

A number of behavioural categories contain *grouped* behaviours. Two types of groupings have been used. *Themed groups* represent a number of behavioural elements combined to describe a particular posture. For example, inattentive posture resting hindlimb is composed of 5 *state* behaviours; resting hindlimb, head level or down, no oral behaviour, no stereotypical behaviour, standing at the middle or back of the stable. *Non-themed groups* represent a number of very similar behavioural elements that may be easily confused, leading to inaccuracies, i.e. hindlimb movement includes kicking, stamping, hindlimb lifting and weight-shifting.

An ethogram of equine behaviour, consisting of a detailed description of each behaviour to be monitored, was generated through the examination of current literature. Tables 2.1 and 2.2 show general ethograms for undisturbed and evoked behaviours. Detailed ethograms can be found in Appendix One. The production of a detailed and clearly defined ethogram is of paramount importance, minimising confusion between similar behaviours.

Type	Category	Behavioural Elements
State	Locomotive	Lateral, sternal or total recumbency; standing bearing weight on all four legs; standing bearing weight on three legs and resting either near hind or fore or off hind or fore; walking forwards or backwards; circling; walking circles
Event	Locomotive	Kicking; stamping hind or foreleg; lifting hind or forelimb; striking; pawing with forelimb; scratching head with hindlimb; pushing part of body against object; rolling; stretching to urinate; shaking body; skin twitching; stretching hindlimb; weight shifting hindlimb; lying down; getting up
State	Attention	Alert; alert and exploring; resting; sleeping
State	Head Position	Head above (up), below (down) or level with withers, head low (down+level)
Event	Head Motion	Ear flicking; tossing, nodding, shaking.
State	Ear Position	Ear positioned forward, backwards to the side
State	Tail Position	Tail relaxed, pressed tightly against rump (depressed) or raised
State	Oral	Eating concentrate, forage or bedding; drinking; lower lip tense, relaxed or quivering; licking; biting; smelling, pushing.
State	Other	Flared nostrils; sweating
Event	Oral	Flehmen; yawn
Event	Tail Position	Tail flicking
Event	Stereotypical	Weaving; stall-kicking; wind-sucking; cribbing; box walking
Event	Other	Eye rolling; urinate; defecate
Event	Locomotive	Hindlimb movement
State	Head Position	Head low
State	Locomotive	Recumbency
State	Oral	Exploratory behaviour
State	Locomotive	Inattentive posture, standing with equal weight-bearing; inattentive posture resting hindlimb.

(Silver 1985; Sanford et al. 1986; Matthews 1992; Johnson et al. 1993; McDonnell & Haviland 1995; Owens et al. 1995; Raekallio et al. 1997a; Raekallio et al. 1997b; Dobromylskyj et al. 2000; Clarke 2001; Eager 2002; Taylor et al. 2002b; Price et al. 2003; Rietmann et al. 2004; Ashley et al. 2005)

Table 2.1 General ethogram for undisturbed and detailed behavioural analysis (a detailed ethogram can be found in Appendix 1.1 and 1.2).

Category	Type	Behavioural Elements
Locomotion	State	Stand (equal weight bearing), resting fore/hindlimb
	Event	Kick, stamp, step away, weight shift, fore/hindlimb lift
Head position	State	Head up or down and either straight, turned away from or towards the handler
	Event	Head movement
Ear position	State	Ears backwards or forwards
	Event	ear flick
Attentive	State	Exploring handler
Oral	State	Lick and chew

Table 2.2 General ethogram for interactive behavioural observations (a detailed ethogram can be found in Appendix 1.3 and 1.4).

2.3 RESPONSE TO ANALGESIA

In order to examine hypotheses two and three of the current research, it was necessary to determine the effect of sedative, anaesthetic and analgesic drugs used. As a clinical case-based study involving client owned horses, anaesthetic techniques used were in accordance with contemporary practice. It was, therefore deemed unethical to significantly alter analgesic protocols or to include experimental groups where pain was left untreated. In addition, it was not possible to obtain blood serum samples to determine circulating drug concentrations. Whilst the lack of circulating drug concentrations is a limitation of this study it should be noted that these values do not necessarily relate either to the duration of action or to the effect. Determination of the pharmacological effects of all agents used on equine behaviour was necessary because;

1. It was deemed unethical not to use current drug protocols.
2. Drug effects need to be separated from pain effects.
3. Due to fluctuations in circulating drug concentrations behaviour may be affected differently at different times.

A review of the relevant literature was performed for all agents used in each of the models, in order to predict the duration of action of each of the drugs used as accurately as possible.

2.3.1 Pharmacology of agents used

It should be appreciated that much of the following pharmacokinetic data are generated using healthy conscious horses receiving no other medication. It is probable the data apply less to horses during general anaesthesia because of the perturbation caused by volatile anaesthetics on factors affecting drug disposition, e.g., hepatic and renal blood flow.

2.3.1.1 Detomidine

Detomidine is α_2 adrenoceptor agonist, with a high level of receptor specificity (Hamm et al. 1995) and both sedative and analgesic effects (Moens et al. 2003), making it useful during standing surgical procedures. Detomidine is widely used in equine practice, commonly in combination with butorphanol as they act synergistically to induce profound sedation (Taylor et al. 1988), administration of α_2 agonists alone results in

sedation but unpredictable sudden responses to stimulation can occur (England & Clarke 1996).

Behavioral effects are similar for all α_2 agonists, including lowering of head, drooping eyelids and lower lip, ataxia, relaxation of the penis, reduced awareness and reduced response to stimulation (England & Clarke 1996).

Detomidine has an elimination half-life of 1.2 hours at 80 $\mu g\ kg^{-1}$ IV (Salonen et al. 1989), a duration of sedation between 30 and 90 minutes and a duration of analgesia of between 30 and 45 minutes (Plumb 1999). Experimental studies suggested peak analgesic occurs approximately 15 minutes after administration (Moens et al. 2003), gradually decreasing over a period of approximately 60 minutes. Duration of analgesia varies from 0 to 45 minutes at 10$\mu g\ kg^{-1}$, dependant on experimental model and site of testing (Jöchle & Hamm 1986; Hamm et al. 1995). At higher doses of 60-160$\mu g\ kg^{-1}$, superficial analgesia was present for up to 2.75 hours and deep pain analgesia was detected 3 hours post-administration (Jöchle & Hamm 1986; Stenberg et al. 1986). Lowe and Hilfiger (1986) report effective analgesia for induced colic pain for 45 minutes at 20$\mu g\ kg^{-1}$.

Sedation has been assessed with a large variety of procedures included measuring the change of distance of the nose to the floor, response to external stimuli such as banging a bucket and through subjective clinical assessment. Following doses of 20 to 60$\mu g\ kg^{-1}$, heavy sedation is reported to occur after 3-5 minutes (Stenberg et al. 1986). Duration of sedation at 10-20$\mu g\ kg^{-1}$ is approximately 100 minutes (Jöchle & Hamm 1986; England et al. 1992; Hamm et al. 1995). Recommended clinical doses are 0.01-0.04$mg\ kg^{-1}$ IV or IM or 0.01- 0.02$mg\ kg^{-1}$ when followed by butorphanol (Bertone & Horspool 2004).

2.3.1.2 Xylazine

Xylazine is an α_2 agonist, the administration of which results in sedation, analgesia and muscular relaxation. Xylazine is commonly used for pre-anaesthetic medication before general anaesthesia in horses and helps to reduce central excitement during induction

(England & Clarke 1996). Plumb (1999) reports a plasma half-life of 50 minutes, whilst Dyke (1993) cites a range of 48 to 75 minutes. Following IV administration elimination half-life has been reported to be between 50 – 75 minutes (Garcia-Villar et al. 1981).

The reported duration of analgesia in horses is variable (England & Clarke 1996). At a dosage of 1.1 mg kg^{-1}, dental pain was reduced for 140 minutes (Brunson & Majors 1987), superficial pain was reduced for between 15 minutes (Moens et al. 2003) and 180 minutes (Kalpravidh et al. 1984b). Using experimentally-induced colic pain, analgesia was obtained after 20 minutes (Lowe & Hilfiger 1986) with 1.1 mg kg^{-1} and between 57 (Lowe 1978) and 240 minutes (Kalpravidh et al. 1984b) after 2.2 mg kg^{-1}.

Onset of sedation is rapid, with a peak occurring between 4 and 8 minutes following IV injection of 0.6 mg kg^{-1} (Garcia-Villar et al. 1981). Kalpravidh et al. (1984a) report the onset of sedation after 15 minutes, continuing for up to 105 minutes. After 1 mg kg^{-1}, ataxia is present for 12 minutes. Reaction to touch and general postural changes are evident for a further 120 minutes (Nolan & Hall 1984). Clinically, dose rates of 0.6-3.0mg kg or 1.1 mg kg are used prior to ketamine (Bertone & Horspool 2004).

2.3.1.3 Morphine

Morphine is an opioid, OP3-receptor agonist, producing analgesia, sedation and respiratory depression (Kamerling et al. 1989). The elimination half-life of morphine is between 88 (Plumb 1999) and 97 minutes (Combie et al. 1983) at a dose of 0.1 mg kg^{-1}. During isoflurane anaesthesia the half-life has been reported to be 40 minutes at 0.25 mg kg^{-1} and 60 minutes at 2 mg kg^{-1} (Steffey et al. 2003). Steffey et al. (2003) describe the analgesic affect as 'profound...with minimal myocardial depression'. Significant effects on visceral and superficial pain have been reported for up to 30-40 minutes at 0.66 mg kg^{-1} (Kalpravidh et al. 1984b).

Concerns over reported adverse behavioural effects have limited the use of morphine in horses (Mircirca et al. 2003). Spontaneous locomotory activity in response to morphine administration has been studied by a number of authors. At 0.66 mg kg locomotory activity was heightened for 4-5 hours (Kalpravidh et al. 1984b) with peak activity

occurring 2 hours post-administration (Combie et al. 1979). At higher doses (1.2mg kg^{-1}) locomotory activity remained increased for up to 14 hours and loss of coordination occurred for up to 7 hours (Combie et al. 1979). However, it has been suggested that behavioural effects are not present at lower doses (Mircirca et al. 2003) although this may then reduce the analgesic potential of the drug (Kamerling et al. 1989). It is believed that morphine is less likely to cause behavioural side effects in animals in pain compared to those that are pain-free and that where post-operative locomotory activity is seen at low doses, observers may actually be seeing post-operative discomfort in animals with insufficient pain relief (Mircirca et al. 2003; Clark et al. 2005). In addition, administration of α_2 agonists, such as xylazine used in this study, reduce the behavioural effects of opioid agonists (Corletto et al. 2005).

2.3.1.4 Butorphanol

Butorphanol is an opioid agonist-antagonist, it is a OP3-receptor antagonist and a OP2-receptor agonist. Opioid agonists are centrally acting, producing analgesia and euphoria. Because of central excitement at maximum dose, butorphanol is most commonly used in combination with $\alpha 2$ agonists for short term sedation and analgesia (Sellon et al. 2001).

The elimination half-life of butorphanol is 44.4 minutes at 0.1 mg kg^{-1} (Sellon et al. 2001). However, a significantly longer duration of action of 3-4 hours has been quoted by (Plumb 1999). Onset of analgesic and sedative effect is rapid, occurring 6 -10 minutes following administration (Jöchle et al. 1989). Studies using experimental assessment of superficial pain report a duration of analgesia of between 15 minutes at 0.1mg kg^{-1} (Kalpravidh et al. 1984a) and 60 minutes at 0.22 mg kg^{-1} (Kalpravidh et al. 1984b). Using visceral pain models the analgesic effect ranged from 15 minutes at lower doses (Kalpravidh et al. 1984a) to 240 minutes at 0.22 mg kg^{-1} (Kalpravidh et al. 1984b). Duration of effective sedation has been cited as approximately one hour (Taylor et al. 1988). Behavioural effects of butorphanol administration include ataxia, staggering, shivering, restlessness and sedation (Kalpravidh et al. 1984a; Kalpravidh et al. 1984b; Sellon et al. 2001), however, these effects occur at significantly higher doses

than those recommended (20μg kg^{-1}) when used in combination with detomidine (Compendium of Veterinary Data Sheets, 2007).

2.3.1.5 Phenylbutazone (single and repeat dose)

Phenlybutazone is a non-steroidal anti-inflammatory drug (NSAID) with antipyretic and analgesic activity (Soma et al. 1983), and no know sedative properties (Johnson et al. 1993). The plasma half-life is dose-dependant and varies between 3 to 8 hours (Piperno et al. 1968), with a dose rate of 4.4 mg kg^{-1} (IV) had a 'half-life' of 5.5 hours (Lees et al. 1987). That the half life varies considerably with dosage, route of administration, feeding, drug accumulation, age and breed has been emphasised by Lees and Higgins (1985). Due to the acidic nature of NSAID's they accumulate in areas of inflammation, resulting in a more profound effect in inflamed than normal tissue (Kallings 1993a). In inflamed tissue drug clearance is slower than in plasma. This results in a relatively short plasma half-life which may not be directly related to its duration of action (Lees & Higgins 1985).

Clinical observation suggests an active duration of greater than 24 hours after a single dose (Dun 1972). Horses suffering from chronic laminitis pain received relief for up to 24 hours following a single intravenous dose (4.4 mg kg^{-1}) with relief peaking at 6 hours (Owens et al. 1995). Post-operatively, the time, before further analgesia was required, was 8.4± 4.6 hours in horses after an initial dose of 4 mg kg^{-1} IV (Johnson et al. 1993).

Repeated dosing can result in drug accumulation and a dose-dependant peak plasma concentrations occurs between 2 and 6 hours post-administration (Gerring et al. 1981). Clinically, chronic digital pain, induced by navicular syndrome, was improved for at least 24 hours and up to 30 hours following 4 days of repeat dosing at 4.4 mg kg^{-1} IV (Erkert et al. 2005). A *Per os* (PO) dose rate of 2.2 -4.4 mg kg every 12 -24 hours is recommened (Bertone & Horspool 2004).

2.3.1.6 Flunixin meglumine

Flunixin is a peripherally acting, anti-prostaglandin, non-steroidal anti-inflammatory drug. Along with phenylbutazone, flunixin is the most commonly used analgesic agent in the horse (Sellon et al. 2001) and as with phenylbutazone, the plasma half-life of flunixin is not directly related to the duration of its clinical action (Kallings 1993b). Values for the plasma half-life have been reported to occur between 96 (Houdeshell & Hennessey 1977) and 146 minutes (Semrad et al. 1985) at 1.1 mg kg^{-1} and after about six hours at a dose of 2.2mg kg^{-1} (Soma et al. 1988). However, flunixin can persist in inflammatory tissue for approximately 16 hours (Landoni & Lees 1995).

The onset of analgesia occurs after approximately 2 hours, with peak responses at 12 hours after administration of flunixin (Houdeshell & Hennessey 1977). One study found limited or no analgesic effect on experimental superficial and visceral pain after a dose of 2.2 kg mg^{-1}, in another study, the duration of relief from clinical colic was between 6-8 hours after a dose of 1.1 mg kg^{-1} (Vernimb & Hennessey 1977). Analgesic activity has been reported to persist for up to 30 hours (Houdeshell & Hennessey 1977), however, clinical experience suggests a duration of action of less than 24 hours (Soma et al. 1988). The recommended clinical dose rate for IV, IM and PO administration is 1.1 mg kg^{-1} (Bertone & Horspool 2004).

2.3.1.7 Lidocaine

Lidocaine or lignocaine is a local anaesthetic agent, blocking sodium ion channels and preventing the passage of sodium ions through membrane pores, hence preventing the initiation and propagation of action potentials (Mason 2004). Lidocaine has a relatively short elimination half-life of 48.4 minutes at 0.6 mg kg^{-1} (Kristinsson et al. 1996). Duration of action has been recorded between 30 and 90 minutes after subcutaneous injection of 10 and 40 mg (Harkins et al. 1998), with a rapid onset of approximately 4 minutes (Grubb et al. 1992).

2.3.1.8 Ketamine

Ketamine hydrochloride is a dissociative anaesthetic, exerting a generalised depressant effect on the CNS, acting as an antagonist of the NMDA receptor (Mason 2004). Once

bound to the NMDA receptor, ketamine does not inhibit the binding of glutamate (primary excitatory neurotransmitter) rather it blocks the transmission of sodium and calcium ions that would normally occur following glutamate activation (Carvey 1998). Administration of ketamine results in dose-dependant loss of consciousness and short duration analgesia in the horse (Kaka et al. 1979). Ketamine has a reported half-life of 1 hour (Plumb 1999). Most studies are based on epidural administration of ketamine and so are irrelevant to the protocols used in the chapters of this thesis.

2.3.1.9 Diazepam

Diazepam is a benzodiazepine, which induces relaxation of skeletal muscle through the facilitation of the action of the neurotransmitter gamma-aminobutyric acid (GABA) (Macleay 2004). Benzodiazepines bind to a specific site on the GABA chloride channel, known as the benzodiazepine receptor. This binding enhances the action of GABA on the channel, increasing chloride conduction (Mason 2004). Diazepam is commonly used in equine veterinary practice as a muscle relaxant and anticonvulsant, primarily in combination with other agents for anaesthetic induction. Research in horses shows a large variation in elimination half-life from between 2.5 to 21.6 hours (Shini 2000). However, the effects of metabolite accumulation renders serum values useless (Plumb 1999). Behavioural effects in the horse have been shown to occur in a dose-dependant manner and include a fixed gaze, muscle fasciculations of the face and neck and at higher doses, ataxia (Muir et al. 1982). These changes continue for between 20 and 60 minutes at low doses (below 0.1 mg kg^{-1}) and for up to 4 hours at higher doses (0.4 mg kg^{-1}). Clinical doses range from 0.05 – 2.0 mg kg^{-1} IV (Bertone & Horspool 2004).

2.3.1.10 Halothane

Halothane is a dose-dependant, central nervous system depressant and gaseous anaesthetic, causing unconsciousness and, therefore, analgesia. The mechanism of action of inhalational anaesthetics is not well understood (Mason 2004). Metabolism is not significant in elimination of inhalational anaesthetics, as the primary route for washout is via the lungs (Mason 2004). Time to standing recovery was 38 minutes following 1 hour of halothane anaesthesia (Whitehair et al. 1993).

2.3.1.11 Nitrous Oxide

Nitrous oxide (N_2O) is not potent enough to act alone as an anaesthetic agent in animals, however, when used in conjunction with other agents (such as halothane), N_2O reduces the minimal alveolar concentration (MAC) required for the maintenance of surgical anaesthesia (Testa et al. 1990). This reduces adverse cardiovascular and pulmonary effects and allows a faster recovery (McKelvey & Hollingshead 2003). In the study reported here, it was used to facilitate uptake of halothane and to improve induction of inhalational anaesthesia.

2.3.2 Limitations of reliance on published literature

Duration of analgesia is notoriously difficult to assess. The majority of studies are based on experimental assessment of pain in which the latency of a simple reflex response is used to assess increases in pain threshold, again using a simple reflex response as an end point. Whilst these experimental studies may be of use in the comparison of analgesic agents, the relationship of this experimental pain to clinical pain is unclear. Commonly, responses to analgesics vary significantly with the type of test and the site of study (such as pelvic or thoracic limb) (Jöchle & Hamm 1986; Hamm et al. 1995). Subtle differences in testing procedures can produce quite different responses (Moens et al. 2003). It is also recognized that experimental pain is very different from that experienced in a clinically setting where a number of types of pain with differences in phase and duration, can occur simultaneously.

The degree and duration of sedation is traditionally assessed subjectively, and horses are assigned a clinical "score". Results are difficult to interpret as none, or only limited descriptions of the variables used to make the assessment are provided. Responses to poorly-repeatable external stimuli, such as banging a bucket (Jöchle & Hamm 1986), are also used. However, no attempt to validate these scoring systems has been made and neither inter- nor intra-observer reproducibility has not been assessed.

As previously mentioned, it should be appreciated that these experimental data are taken from conscious, pain-free animals administered a single agent. This is not as close an

approximation to the clinical environment in which these drugs are generally used as would be preferred.

2.3.3 Drug protocol and predicted duration of action

2.3.3.1 Standing surgical castration with local anaesthesia and sedation

Horses were sedated with detomidine (10μg kg^{-1} IV) and butorphanol (20μg kg^{-1} IV) in combination. Examination of the literature suggests the maximum duration of analgesia associated with detomidine at 10μg kg^{-1} IV is approximately 60 minutes. Sedative properties are reported to be longer in duration, approximately 100 minutes. Therefore, it was assumed that detomidine will have minimal effects at the first sample point, two hours post-operatively as can be seen in figure 2.2.

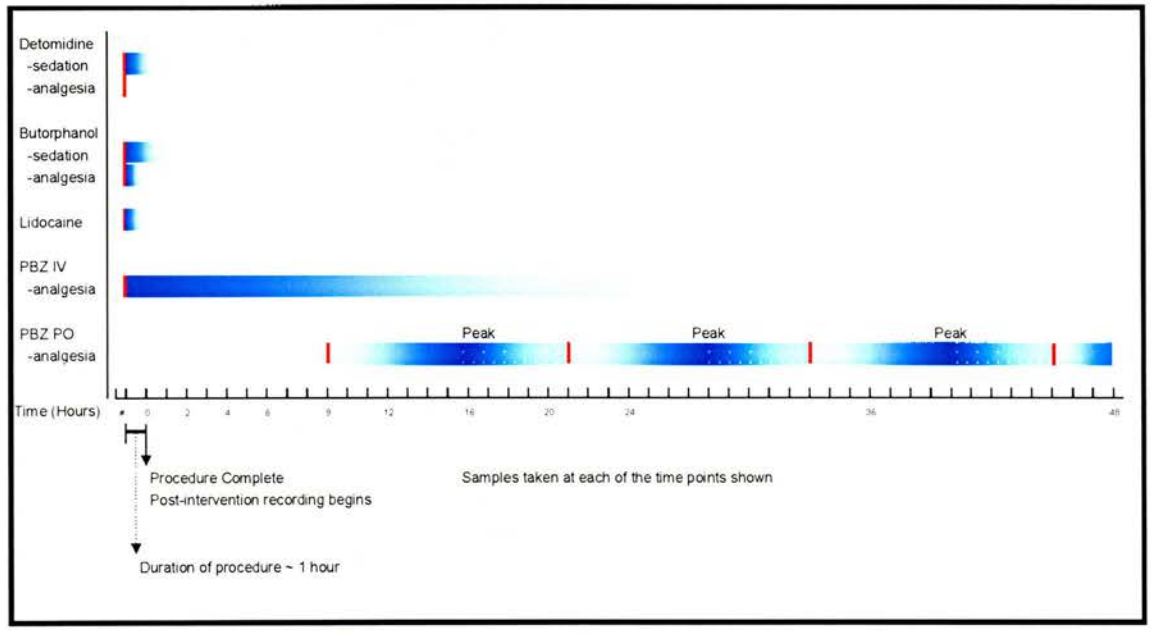


Figure 2.2 Prediction of the duration of action of all drugs used for castration with standing surgical anaesthesia and control horses subject to standing surgical anaesthesia. The red bars indicate time of administration of each agent. The blue bars represent the predicted duration of action with dark blue showing peak and light blue showing minimal activity.

Studies of the action of butorphanol have generally used a higher dose (0.22 mg kg^{-1}) than that used in this study (0.02 mg kg^{-1}). Research has suggested the duration of analgesia after 0.22 mg kg^{-1} to be between 60 and 240 minutes, with sedation lasting for up to 60 minutes. It is assumed that the sedative and analgesic effects of butorphanol, as with detomidine, will be minimal at the first experimental time point. Local anaesthesia

(20ml 3% of lidocaine + 12.5μg kg^{-1} epinephrine) was infiltrated into each spermatic cord. The literature suggests a duration of action of approximately 45 minutes at this dose.

Intravenous phenylbutazone (4.4mg kg^{-1} IV), see Fig 2.2, was administered pre-operatively. Peak responses occur approximately six hours after administration, with analgesic effect decreasing over a 24 hour period. Post-operatively, horses received phenylbutazone (4.4mg kg^{-1} PO) BID. Repeat dosing can result in drug accumulation. Peak responses were predicted at between two and six hours post-administration. In order to obtain data for times of minimal drug activity, data should be collected immediately before re-administration.

2.3.3.2 Castration under general anaesthesia

Pre-anaesthetic medication, injected immediately after horses entered the induction box was intrvenous xylazine (1.1 mg kg^{-1}). Research suggests a relatively short duration of action at this dose. With a surgery and recovery time of approximately two hours, it was assumed that xylazine would not be acting as the horse was returned to the stable (time 0).

Intravenous flunixin meglumide (1.1 mg kg^{-1}) was administered five minutes before anaesthesia. As discussed previously, research findings are extremely varied, ranging from 7 to 30 hours. In this study, it was assumed that the analgesic effects of flunixin were present for approximately 24 hours following administration.

Anaesthesia was induced five minutes later by intravenous injection of ketamine (2.2 mg kg^{-1}) and diazepam (25 μg kg^{-1}) in combination. Following endotracheal intubation, horses were transferred to the operating theatre. It was considered that ketamine was not having a significant effect at time 0, i.e. approximately two hours after the surgery and recovery time. Diazepam, at the low dose administered here, was also not considered to be having a significant effect at time 0.

Inhalational anaesthesia was maintained on a circle breathing system (Large Animal Control Centre, Draeger Medical, Herfordshire, U.K.) with halothane in oxygen (50%) and nitrous oxide (50%). End tidal halothane concentration was maintained at 0.8 – 1.0%. Nitrous oxide was discontinued after 10 minutes and oxygen (100%) used doe the rest of the time.

At the dose of morphine administered here (0.12 mg kg $^{-1}$ IV), analgesia was expected to last for approximately 30 minutes. Due to the low dose, no adverse behavioural responses were expected.

Post-operatively, phenylbutazone (4.4 mg kg $^{-1}$) was given orally, twice daily, at 2000h and at 0800h and 200oh for the remainder of the study. As for standing surgical castration, the peak responses are for repeat dosing were predicted to occur between two and six hours post-administration and points of minimal drug activity occured immediately before re-administration. Assumptions made from this information are shown diagrammatically in figure 2.3.

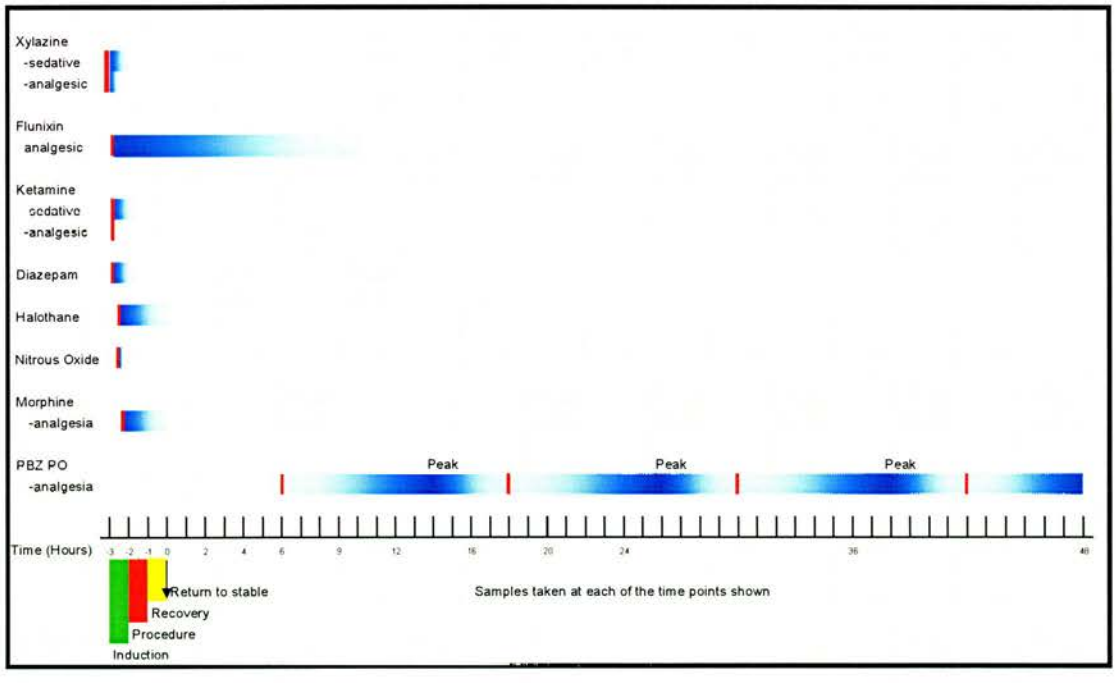


Figure 2.3 Prediction of the duration of action of all drugs used for castration with general anaesthesia and control horses subject to general anaesthesia. The red bars indicate time of administration of each agent. The blue bars represent the predicted duration of action with dark blue showing peak and light blue showing minimal activity.

2.3.3.3 Laminitis

Laminitic horses were treated with repeat doses of PBZ every 12 hours at 0800h and 2000h. The therapeutic goal of this regime being the maintenance of an analgesic 'steady state'. The plasma PBZ half-life is variable and dependant on dose, route of administration and the metabolism of individual animals (Kollias-Baker & Cox 2004). With a reported maximum half-life of 8 hours (Lees & Higgins 1985) and dose-dependant peak plasma concentrations occurring between 2 – 6 hours following administration when repeat dosing (Gerring et al. 1981), However, it is likely that somme fluctuation in phenylbutazone activity may occurred between sample points. The 0600h sample point should occur at a minimum plasma PBZ concentration while the 1400h and 2200h sample points should occur at peaks in PBZ concentration.

Soma *et al.* (1983) found that repeating oral PBZ doses twice daily could provide a in stable plasma PBZ concentrations over a period of 4-5 days. However, accumulation of PBZ has been found in association with repeated dosing over 4 days and when using the highest dose administered here (Gerring et al. 1981). It should also be noted that the acidic nature of non-steroidal anti-inflammatory drugs may also lead to accumulation in sites of inflammatory action which also tend to be acidic (Lees & Higgins 1984). Therefore, drug accumulation within the hoof (in this case) may occur. This suggests that repeated PBZ administration leads to drug accumulation, and an increasing analgesic action over the duration of the study.

2.5 STATISTICAL METHODOLOGY

The development of valid scale for the assessment of pain requires a thorough approach. Initially, appropriate indices need to be identified through objective study of behavioural responses. External factors such as environment, time, time of day and drugs may have an effect on different behaviours in different ways. Consequently, objective identification of potential confounding effects is necessary when generating an accurate and reliable pain assessment protocol. In addition, pain is a complex multifaceted phenomena and it is generally appreciated there is no single marker for the accurate assessment of pain severity. The identification of combinations of important behaviours may improve accuracy of assessment. It is also important that an assessment

be succinct and so the elimination of redundant indices may improve ease of use and overall reliability. Effective testing of the experimental hypotheses required the implementation of a number of statistical techniques. Univariate analysis techniques were employed in the identification of overall differences between groups, effects of environmental factors and the response to analgesia. Multivariate analysis was used to highlight the importance of particular behaviours, to determine the potential ability of a number of variables to discriminate between groups and to identify optimal combinations of variables.

2.5.1 Univariate analysis

State behaviours were recorded as a proportion of the total sample time, whereas event behaviours were recorded as a frequency. In some cases, a behaviour may have been affected by another, or slight variations in management routine may have affected behaviour. For example, in laminitic and SS castrate groups, variations in feeding may have affected head position. In this case, head position was only examined when the animal was not feeding. Behaviours showing majority '0' values were excluded from analysis.

In his textbook '*Statistical Computing*', Crawley (2002) states

'Statisticians have an agreed convention about what constitutes 'unlikely'; they say an event is unlikely if it occurs less than 5% of the time.'

Therefore, in all cases $P<0.05$ was taken to indicate statistical significance for the purpose of this thesis. However, it is recognised that the use of $P<0.05$ is debated, especially in association with multiple comparisons. All statistical results described here were interpreted with caution and considered with regard to the biological/clinical significance of the finding as opposed to the absolute statistical significance.

Univariate statistical analyses were performed using The R Project for statistical computing (R Foundation, http://www.r-project.org).

2.5.1.1 General analysis

General analysis was performed to examine overall trends, effects of a number of variables and the interactions between variables on the data. This analysis was performed on undisturbed, direct observation and interactive datasets for all three models. In order to account for the repeated sampling of the same horses over time, analyses were carried out using generalised mixed effects models (Pinheiro & Bates 2000). These models allow the inclusion of random (influence experimental variation) and fixed ('unknown constants to be estimated from the data') effects (Crawley 2002). In castration and laminitic models horse ID (individual identification for each animal) was entered as a random effect. Univariate analysis examined the effect of a single fixed effect. In castration models experimental group (castrate or control), treatment (baseline or post-intervention) and time (number of hours post-intervention and equivalent baseline times) were entered as fixed effects. In the laminitis model experimental group (laminitic or control), time (number of hours from start of study), day (day 1-5) and time of day (time at which each sample was taken-0600h, 1400h or 2200h) were entered as the fixed effects. Multivariate analysis examined the interactions between fixed effects (to test whether any changes with treatment, time, time of day etc were due to which group the horses came from).

Proportion data (state behaviour) were analysed using binomial errors. In most scenarios, data were proportions of 3600 seconds (1 hour sample duration). However, as previously described, some behaviours were adjusted for the affects of husbandry or other behaviour, resulting in disparity in the sample duration. Interactive behavioural tests also varied in duration. One variable could therefore, be a proportion of, for example, 0.5 of 3600 seconds and another 0.5 of 900 seconds. Data created as a proportion of 3600 seconds are a more accurate representation of behaviour than that taken from 900 seconds.

In order to account of unequal weighting of proportions, algorithms for analysis of proportional data included a response vector containing the number of successes (seconds spent performing) and failures (seconds spent not performing). This allowed the calculation of a new weighted response variable (Crawley 2002) which was applied

in mixed effects models. Weighted variables were also used in the generation of graphs. For graphical presentation of these data, standard error bars were adjusted for the binomial distribution.

When fitting continuous data to mixed effect models it is assumed that the distribution of the residuals is normal. Therefore, prior to analysis of continuous frequency data (event behaviour) the distribution of residuals was checked. If data were found to contravene the assumptions of the model, square root transformation was applied as this seemed the most appropriate. In cases where insufficient data made it impossible to achieve a normal distribution of residuals through transformation, occurrence and level of occurrence analyses were used. Occurrence analysis used mixed effects models to determine whether there was a significant difference in the number of times a behaviour occurred, irrespective of the frequency at which it occurred. Binomial errors were used as responses were now either '0' or '1'. Fixed and random effects were as previously described.

Secondly, level of occurrence analysis tested whether or not there was a difference in the frequency of behaviour, considering only samples in which behaviour occurred. Subsets of data were created in which all zero values were discounted. Mixed effects models were applied using fixed and random effects as before.

For the purposes of graphical presentation of square root transformed data, descriptive statistics were calculated on transformed data and then back transformed to allow presentation on a normal scale.

Post hoc analyses were performed when experimental variables with more than two levels (such as day or time of day) were shown to have a significant effect. Analysis aimed to determine which level (e.g. time of day) was producing the effect. Initial analysis was repeated on new datasets, created by removing subsequent levels of data. For example, when examining effect of time of day, data would be divided into three new datasets;

1) Data from sample points 1400h and 2200h

2) Data from sample points 0600h and 2200h
3) Data from sample points 0600h and 1400h.

2.5.1.2 Time point analysis

Time point analysis investigated the difference between experimental groups at specific post-intervention (in castrate models) time points. Time points of interest include those where the sedative and analgesic effects of pre-operative drugs have returned to baseline and time points at which levels of post-operative analgesic are at a minimum. These points were chosen relative to pharmacological information gathered in section 2 and with reference to figures 2.2 and 2.3.

Time point analysis for castration models compared between castrate and control groups at specifically selected, post-intervention time points. Distribution of the residuals was examined for normality. A two sample *t-test* was used to compare between groups that were normally distributed. If this was not possible a Wilcoxon rank sum test was used to compare between groups. Comparisons were also made between post-intervention and equivalent baseline time points in castrate and control groups separately, using a paired two-sample *t-test* or Wilcoxon signed rank test.

As laminitic data was obtained at a number of time points over 5 days, time point analysis for this model was based on datasets created from a specific time point each day. Mixed effects models were used to determine the effect of experimental group, time (duration in experiment) and day on these data.

2.5.2 Multivariate analysis

Univariate analyses enabled the identification of potentially useful behavioural indicators of pain and the effects of external variables. However, these do not permit the examination of the roles of combinations of behaviours in the differentiation between experimental groups. Whilst it is well accepted that accurate assessment of equine pain may rely on a number of variables, for an assessment protocol to be practical, it should consider a small number of important behaviours. The use of these types of analyses can help determine the relative importance of certain indices or groups of indices

(Molony & Kent 1997, Molony et al. 2002) and eliminate unnecessary parameters (i.e. those that do not contribute to the discrimination between groups) to provide an efficient system of assessment. In this study two types of multivariate analysis were applied, classification tree-based models and stepwise linear discriminant analysis.

2.5.2.1 Classification tree-based models

Classification tree-based models (hereafter 'tree-models') provide an exploratory technique for examination of the hierarchical structure of data, and are a useful alternative to multivariate analysis. The tree branches are determined by *recursive partitioning* (Clark & Pregibon 1997), which successively splits the data into specified groups (i.e. laminitic or control) using the predictive factors (time spent performing a specific behaviour, i.e. standing).The initial division is formed from the behaviour which forms the most effective division between experimental groups. Following the initial division, one subset is considered (i.e. more than 50% of time spent standing) and the behaviour best showing discrimination between groups selected to form the next subset. Recursive partitioning continues for smaller and smaller subsets of data, until no further differentiation between experimental groups is possible. Subsequent divisions are determined by importance of behaviour within a specific subset and may indicate different behaviours for different parts of the dataset. The analysis is finally outputted in graphical form.

One problem is that these analyses do not support repeated measures and consequently analysis could only be performed on one single observation at a time. Due to the financial and time constraints, the sample size used in the current study ($n = 10$) and the possibility of large individual variation (impossible to control for the effects of previous experience in the equine population) it was unlikely that tree-model analysis would consistently highlight the same pattern of behaviour for each time point. In addition, due to the phasic nature of clinical pain and the effects of diurnal variation, importance of behaviours in the discrimination between groups might vary among time points. Taking these factors in to account, tree-models were drawn for each post-intervention time point and analysis was based on the examination and comparison of tree-models

generating a group of 'important' behaviours. Tree- models were drawn using The R Project for statistical computing.

2.5.2.2 Stepwise linear discriminant analysis

Discriminant analysis determines variables that distinguish between known groups, for instance, laminitic and control horses. Whilst tree-based models consider variables hierarchically, discriminant analysis simultaneously examines a combination of variables which best discriminate between groups. Where more than two groups are present canonical discriminant analysis is used, however, as the studies reported here included only two experimental groups, linear discriminant analysis was used. A forward stepwise technique was used in this analysis. This technique adds behavioural variables one by one to the discriminant analysis model with the aim of finding a subset of behavioural variables which are all associated with pain. Initially, the stepwise technique determines which of many variables most accurately determines between two known groups. This variable begins the model. At the second 'step' the analyses determine which, if any, variables improve the discrimination between groups. If a variable is found to contribute to the discrimination, it is added to the model. Behaviours that do not contribute to the discrimination between groups are therefore not included in the model.

As with tree-models, these analyses will not account for repeated measures. Time points for analysis were selected as described in time point analysis for SS and GA castrate models. In the laminitic model time point 0600h on day two was selected for analysis, as a time point of minimal analgesic action, at which horses first should become habituated to their environment. These analyses were carried out using SPSS statistical software (SPSS, Chicago, USA).

2.5.3 Experimental Sample Size

The experimental sample size needed for this study was determined using a combination of approaches including power analysis of preliminary data, examination of the sample sizes used in other similar studies and consideration of practical and financial

constraints. It was necessary to determine a sample size that would allow reliable statistical analysis, at the same time as also being practical and achievable.

Power analysis was performed on preliminary data from horses undergoing castration. Key behaviours, such as time spent lying, were selected based on results of preliminary analysis on this data (Eager 2002). The recommended sample sizes varied from two to 32 animals.

Reviewing the scientific literature aimed to determine sample sizes used in similar studies. Pritchett et al. (2003), for example, compared a control group of 10 horses with a surgical group of 7 horses to identify behaviours associated with pain. Raekallio et al. (1997b) used a sample size 13 when comparing different methods of assessing pain in equines following orthopaedic surgery. Comparing horses undergoing arthroscopy to clinically normal control horses to identify potential behavioural indicators of pain, Price et al. (2003) used an $n = 6$.

Working with equines was also an extremely limiting factor in the practical implementation of a required sample size. For instance, purchase costs (for animals undergoing Home Office licensed work) were extremely high and animals were difficult to source. Maintenance of equines is also costly and time consuming. Additionally, in order to overcome some of the above mentioned financial and sourcing difficulties and to produce and ethically acceptable protocol, it was decided to work, as far as possible, with clinical cases. This methodology itself provided limitations due to the availability of suitable cases.

Combining the finding of these approaches suggested a sample size of ten would fulfil both scientific and practical criteria. This was achieved in the standing castration and control group and in the general anaesthesia control group. In the general anaesthesia castration group, ten horses underwent surgery, however, due to post-operative colic; one horse was removed from the dataset. In the laminitis group, lack of suitable clinical cases within the experimental timeframe meant that a group size of seven laminitic and seven control horses was achieved.

5.5.4 Assessment and monitoring of intra-observer reliability

Intra-observer variation (often termed repeatability) describes variation in the scoring of the same thing by one observer. This *observer drift* may result from changes in measuring technique (often thought of as improvements), changes in perception of the observer or the observer becoming more careless as the study proceeds. Inter-observer variation (also known as reproducibility) represents the differences in measurements made by different observers measuring the same thing (Martin & Bateson 1993).

During the course of this work one main observer (RAE) was involved in the analysis of video data. Two other observers were involved to a lesser extent. Assessment and monitoring of both inter- and intra-observer reliability was therefore of importance and the below protocols were used.

Protocol for assessment of intra-observer reliability

1. Selection of 3 x 15 minute test video sequence
2. Analysis of test video sequences and careful checking to give 'gold standard' results for these sequences.
3. Monthly reanalysis of test video sequences by observer
4. Visual comparison of 'gold standard' and reanalysis results

Protocol for induction of a new observer and assessment of inter-observer reliability

1. Familiarisation with the ethogram and The Observer™
2. Discussion and standardisation with 'gold standard' observer (RAE)
3. Practice and standardisation
4. Analysis of test video sequences
5. Visual inspection of the raw data and comparison to 'gold standard' recording results.
6. If discrepancies were identified the observer would discuss these with the 'gold standard' observer, reread the ethogram and practice/standardise until both observers agreed

2.6 ETHICAL JUSTIFICATION FOR THE STUDY

Whilst it is necessary to study the physiology of pain and methods for pain assessment in animals in order to promote optimal animal welfare, the use of all animals in research studies should be justified. The study of pain may be more ethically acceptable if the effects of clinical disease, accidental trauma or surgical treatment are examined (Rutherford 2002). The justification for the use of castration as a model of pain is partly reliant on the fact that this routine, elective procedure would have been performed at the client's request, not as an experimental procedure. As is the case in the studies reported here, the study of clinical pain often precludes veterinarians leaving pain untreated and therefore the inclusion of negative controls. In this study, it was not considered ethical to withhold analgesia from horses undergoing surgery, and clinical drug protocols were as generally used in the hospitals included. However, preliminary studies suggest that significant alterations in behaviour could still be seen with the level of analgesia achieved (Eager 2002).

The use of spontaneous disease (laminitis) for the study of a chronic pain state is easier to justify ethically than the induction of an experimental pain state. The animals used within the current study were clinical cases admitted for management of naturally occurring, laminitis. Clinical treatment of these cases was unaltered and euthanasia was performed where necessary. However, whilst the use of spontaneous disease may be ethically acceptable, the inability to strictly control variables reduces the validity of data collected (Rutherford 2002). Standardisation of disease state (e.g. duration, severity of pathology) is not possible and severity of pain may have altered within individual animals during the course of the study.

It is hoped that these studies, will help develop a better understanding of equine pain and its assessment. Only through the improvement of pain recognition can optimal pain management and hence optimal welfare be achieved. The development of a valid, sensitive and reliable technique for the assessment of equine pain will be of use in the evaluation of analgesic efficacy and hence lead to further improvements in equine welfare.

CHAPTER THREE

BEHAVIOURAL RESPONSES TO CASTRATION AND SHAM CASTRATION UNDER STANDING SURGICAL ANAESTHESIA

3.1 INTRODUCTION

The recognition, evaluation and alleviation of animal pain associated with injury or disease is a fundamental objective of veterinary medicine. Inadequate provision of analgesia may not only result in suffering, prolonged recovery and increased hospitalisation times, but increased morbidity and mortality. Despite the obvious importance of pain management, there appears to be a general lack of consensus within the U.K. veterinary profession regarding pain severity associated with specific equine conditions (Price et al. 2002). In the same study, the authors noted that heart rate and 'demeanour' were cited as the most commonly used indicators of post-operative pain in the horse.

It is assumed that extensive clinical experience can provide veterinarians with tools for the assessment of equine pain, however, these observation are fundamentally subjective and therefore not exempt from attitudinal influences. In addition, where care is provided by multiple personnel, possibly including veterinarians, nurses and owners, standardisation and hence accurate monitoring of pain is problematic. Where precise description of parameters to be assessed is not available, knowledge transfer between experienced and inexperienced clinicians is hindered.

These issues are not helped by the relative paucity of objective research in the field of equine pain. In contrast, techniques for evaluation of pain and discomfort have been extensively investigated in laboratory animals (Roughnan & Flecknell 2000; Roughnan & Flecknell 2001), farm animals (Molony et al. 1995; Molony et al. 1997; Molony & Kent 1997; Thornton & Waterman-Pearson 1999), dogs (Hardie et al. 1997; Hansen et al. 1997; Conzemius et al. 1997; Holton et al. 1998; Firth & Haldane 1999; Holton et al. 2001) and cats (Cambridge et al. 2000). The majority of work in the horse uses either quantitative sensory testing techniques for the assessment of analgesic efficacy, or

arbitrary, un-validated behavioural and physiological parameters, results of which may have little application to the assessment of clinical pain.

Uni-dimensional pain assessment scales are often used without proper validation (Holton et al. 2001). Attempts at the generation of multi-factorial assessment protocols have aimed to improve accuracy through the inclusion of a number of parameters. However, inclusion of a specific parameter often appears to rely on subjective opinion or anecdotal evidence. Levels of pain severity are often indicated through graded responses such as an increase in frequency of specific behaviours. However, these scores are again, subjectively assigned with no evidence of a correlation with pain severity. Furthermore, the summation of a number of behavioural responses does not include examinations of the importance of a specific behaviour.

The current study aimed to objectively identify potential behavioural indicators of pain in the horse. Castration is one of the most common surgical procedures in the horse (Schumacher 1996), performed to minimise unwanted sexual behaviour and facilitate management (Houpt 1999). There are a number of different approaches to this surgery, with the protocol selected often determined on the preference and experience of the clinician (Mason et al. 2005). In the current study, castration was considered to be a good model of acute post-surgical pain due to standardisation of the surgical insult and the possibility of obtaining pre-operative, 'pain-free' baseline data. The relative abundance of clinical cases also made castration a feasible option for study.

Castration can be performed under standing surgical anaesthesia (SS) and general anaesthesia (GA), both of which are commonly used in U.K. equine practice (Price et al. 2005). SS castration is undertaken in the conscious, sedated horse given local anaesthesia (Green 2001). GA castration is performed in dorsal recumbency in aseptic hospital conditions, or in some circumstances, in the field. General anaesthesia is often thought a preferential method of restraint, considering it is impossible to sedate some horses adequately for safe completion of the procedure (Mason et al. 2005). However, general anaesthesia in the horse is associated with a mortality risk of 0.9% (Johnston et al. 1995), with a recent study identifying a mortality rate of 1.04% in association with

GA castration (Mason et al. 2005). This risk is reduced when castration is performed in the standing, conscious horse (Nolan & Hall 1984). Despite this risk, castration under general anaesthesia has been associated with lower risks of post-operative complications, including severe haemorrhage or eventration of the intestines (Mason et al. 2005). In some situations, such as in more mature horses, in cryptorchid animals and when the horse is too small to castrate standing, GA castration is required. Cost implications may also affect the approach used, with general anaesthesia being significantly more expensive than standing surgical anaesthesia (Mason et al. 2005).

In the following study, castration was performed under standing surgical anaesthesia, a procedure commonly performed in the field (Price et al. 2005). The performance of this procedure in the animal's home environment was considered to provide a model of clinical pain, without including the effects of hospitalisation stress, which may significantly alter both behaviour and physiology. In addition to the primary aim, a further objective was to elucidate the behavioural effects of sedation in order that any confounding effects in a pain assessment protocol could be addressed in later studies. The null hypotheses specifically addressed in the current study were;

1) Behaviour will not be altered in association with castration pain.
2) There will be no effects of sedative and analgesic drugs on behaviour.
3) There will be no significant difference between times of maximal and minimal analgesia.
4) Behaviour will remain consistent over time.
5) Behavioural parameters will not accurately discriminate between castrated and control horses.

3.2 METHODOLOGY

3.2.1 Subjects

Ten clinically normal, thoroughbred (TB) colts (age: 2.4 years ± 0.5 (SD)) presented to an independent equine veterinary practice for 'in the field' castration under standing surgical anaesthesia. Informed client consent was obtained prior to participation in the study. The control group consisted of ten clinically normal, TB/warmblood geldings (age: 3.4 years ± 1.5 (SD)) from a research population at the Royal (Dick) School of Veterinary Studies (R(D)SVS). Subject details can be found in Appendix 2.1.

3.2.2 Maintenance

All horses were maintained on their home yard during the experiment and were fed in accordance with usual protocols for the specific training facility. In all cases water was provided *Ad Libitum*. The stable size was 15.1 m^2 ± 5.2m (mean ± SD) in castrate horses and 20 m^2 in control animals.

3.2.3 Anaesthetic and Analgesic Protocol

Weight was determined by visual estimation by an experienced clinician. Sedation was obtained by intravenous injection of detomidine (10μg kg^{-1} - Domosedan, Pfizer Ltd) in combination with butorphanol (20μg kg^{-1}- Torbugesic, Fort Dodge Animal Health). Pre-operative analgesia (phenylbutazone) was administered intravenously (4.4mg kg^{-1}- Equipalazone, Arnolds Veterinary Products). Local anaesthesia was administered via subcutaneous injection of 20ml 3% lignocaine + 12.5μg kg^{-1} adrenaline (Norbrook Laboratories (GB) Ltd, Cumbria, U.K.) into each testicle. Castrate horses received the full anaesthetic protocol whereas control horses received sedation and analgesia but not local anaesthesia (and were not castrated). Post-intervention, castrate and control horses were food deprived for two hours.

3.2.4 Surgical Procedure

In the castrate experimental group, open castration was performed by one of three experienced equine veterinarians. The genital region was thoroughly scrubbed with

chlorhexidine (Hibiscrub – SSL International plc). An incision was made into each testis through the skin of the scrotum and into the parenchyma. The testis was then pulled through the incision, exposing the epididymis. This tissue was cut using an equine emasculator (SERRA Crowhurst modified - Arnolds Veterinary Products), following which the emasculators were left in place for approximately 30 seconds. The emasculator was then applied to the spermatic cord and *vas deferens*, which were cut and crushed for up to five minutes. The protocol was then repeated for the other testis.

3.2.5 Assessment Protocol

Assessment included undisturbed monitoring of spontaneous (when the animal was on it's own in the stable) and evoked behavioural responses (when a standardised interactive handling procedure was carried out). Details of these procedures can be found in section 2.2. Baseline data were collected one day prior to intervention (castration or sham castration). Time '0' was established as the point at which the veterinarian left the stable in castrate horses and 45 minutes following the administration of sedative drugs in control horses. Point samples of undisturbed, spontaneous behaviour were taken from video tapes at 2, 4, 6, 9, 12, 16, 20, 24, 36 and 48 hours post-intervention and at equivalent baseline times. Evoked behaviour assessments were performed at 6, 12, 14, 36 and 48 hours.

3.2.6 Statistical Analysis

A detailed explanation of all statistical techniques used is given in section 2.5. Three techniques were used to examine data collected from spontaneous and evoked behaviour sampling. In all cases a P value of <0.05 was taken to indicate significance.

1. General analysis examined overall trends in the data to highlight key behaviours in the assessment of equine pain. Further to this, overall examination of data aimed to elucidate the effects of drugs used, time and time of day on behaviour in each experimental group, determining applicability for use in a pain assessment protocol.

2. Time point analysis examined for any differences in behaviour at specific time points. This methodology aimed to validate potential indicators of pain through the examination of times of minimum and maximum analgesia. Due to the effects of post-operative starvation, it was assumed that feeding motivation may be high at two and four hours. Therefore, 6 hours post-intervention was chosen as the first point at which the effects of sedation and starvation would have worn off. 16 hours post-operative was estimated to be the point of maximal analgesia, with 20 hours post-intervention representing point of minimal analgesia.

3. Multivariate analysis employed two different techniques, classification tree-based models (tree-models) and linear discriminant analysis. Tree-models were used to identify those behaviours most important in discriminating between behaviours exhibited by castrate and control horses. Discriminant analysis examined the importance of a number of behaviours considered together. This analysis aimed to identify important *clusters* of behaviours and to remove those behaviours which did not contribute to discrimination between groups.

3.3 RESULTS

Castrate horse number 10 was removed from analysis due to administration of acepromazine in addition to sedative protocol described. There was no significant difference in age between the two groups (P=0.086). Preliminary exploratory analysis examined the data graphically to identify potential behavioural variables indicative of pain and to recognise any effects of variables other than pain. Figure 3.0 shows a selection of the preliminary screening graphs.

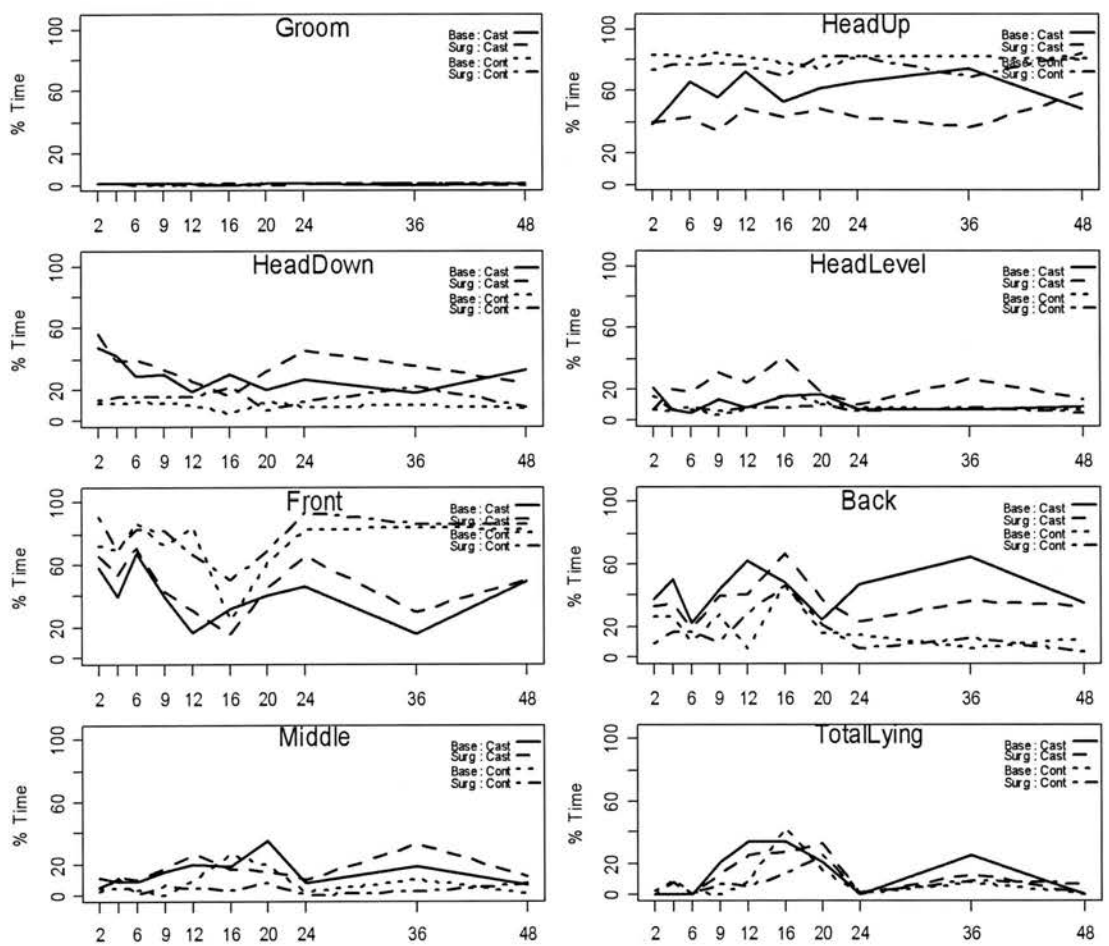


Figure 3.0 Preliminary graphical analysis of individual behavioural variables showing mean proportion of time spent performing individual behaviours by castrated and control groups at baseline and post-intervention periods.

In figure 3.0 the behaviour 'total lying' shows an example of 'no visible effect of either pain (surgery) or sedation'. The lines of the graph representing the four groups (baseline

castrate, baseline control, post-intervention castrate and post-intervention control) all follow a similar pattern and there are no obvious differences between them.

Behaviours potentially associated with pain are identified by a gap between the 'castrate post-intervention' line and the three other lines. An example of this can be seen in the 'HeadLevel' graph in figure 3.0 The 'castrate post-intervention' line lies above the other lines suggesting that following surgery, castrated animals spend more time with their head level to their withers than either castrate animals before surgery (baseline), control baseline or control animals after sedation.

Examining the graph of 'Front' which shows the difference between groups in 'time spent at the front of the box' shows the four lines splitting into two sets of pairs (most predominant between time points 24 and 48). The upper pair of lines represents control horses at baseline and post-intervention and the lower pair of lines represents castrated horses at baseline and post intervention. This suggests that there are fundamental differences between the behaviour of the castrated and the behaviour of the control horses, even before intervention. Further examination of the graph suggests that the time spent at the front of the box is altered following intervention. In castrated and control groups the post-intervention line appears to lie above the baseline data line indicating that the time spent at the front of the box is increased after intervention. As the decrease is seen in both horses undergoing castration (sedation + surgery) and those receiving sedation alone, it is potentially associated with sedation.

Behaviour	Experimental period	Castrate	Control
Total Lying	Baseline	0.16 ± 0.27	0.1 ± 0.2
	Post-intervention	0.12 ± 0.27	0.09 ± 0.22
Head Level	Baseline	0.14 ± 0.19	0.11 ± 0.18
	Post-intervention	0.19 ± 0.28	0.14 ± 0.19
Front of box	Baseline	0.39 ± 0.28	0.7± 0.3
	Post-intervention	0.47 ± 0.32	0.76 ± 0.32

Table 3.0 Descriptive statistics for example behaviours proportion of time spent lying, with head level and at the front of the box. The table shows the mean ± standard deviation for the castration and control groups at baseline and post-intervention periods.

Descriptive statistics confirm these observations, as can be seen in table 3.0. There is very little difference in the castrate and control values for the behaviour 'total lying'. The behaviour 'head level' shows little difference between castrate and control groups at the baseline period. However, the difference between groups at the post-intervention period is greater, suggesting a change in behaviour post-intervention in the castrate group.

Table 3.1 shows a summary of significant results for univariate general analysis of both spontaneous and table 3.2 shows results for evoked behaviours. Complete tables of results (including degrees of freedom, test statistics etc) can be found in Appendix 2.2 – 2.4.

Behaviour	Group	Treatment	Time	Interaction Group*Treatment	Interaction Group*Time
Inattentive	P=0.248	**P=0.005**	P=0.491	P=0.071	P= 0.457
Head low	**P=0.009**	**P<0.001**	P=0.111	**P<0.001**	P=0.703
Exploratory	**P<0.001**	P=0.075	P=0.864	P=0.864	P=0.761
Grooming	P=0.874	**P=0.049**	P=0.998	P=0.646	**P<0.001**
Head up	**P=0.01**	**P<0.001**	P=0.111	**P<0.001**	P=0.704
Head down	P=0.847	**P<0.001**	P=0.232	P=0.247	P=0.476
Head Level	**P=0.008**	**P<0.001**	P=0.184	**P<0.001**	P=0.973
Front	**P<0.001**	**P=0.032**	P=0.999	P=0.933	**P=0.039**
Middle	**P=0.041**	P=0.212	P=0.185	P=0.194	P=0.595
Back	**P=0.001**	P=0.118	P=0.394	P=0.698	P=0.059
Leg move	P=0.697	P=0.199	P=0.961	P=0.357	**P=0.013**
Weight shift*	P=0.851	**P=0.035**	P=0.843	P=0.906	**P=0.004**
Tail flick	P=0.223	P=0.677	P=0.590	**P=0.030**	P=0.743
Head shake≠	P=0.927	P=0.370	**P=0.016**	P=0.945	P=0.673

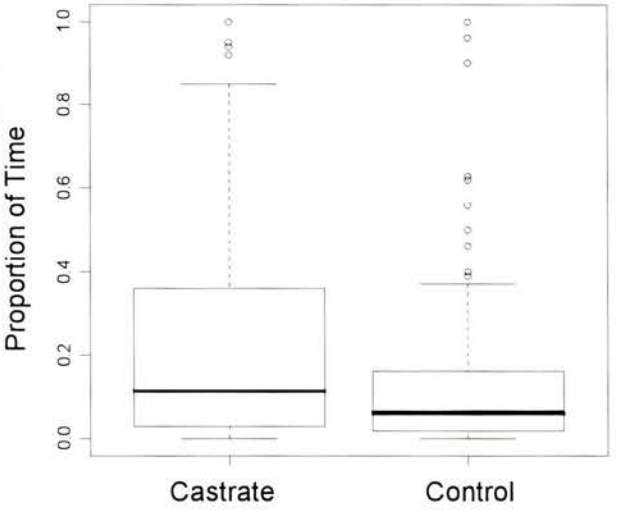
Table 3.1 Summary of univariate results for spontaneous behaviour with * denotes occurrence analysis and ≠ level of occurrence analysis.

Behaviour	Group	Treatment	Time	Interaction Group*Treatment	Interaction Group*Time
Ears back	**P=0.012**	**P<0.001**	**P=0.032**	**P<0.001**	P=0.479
Head up (turned away)	P=0.314	P=0.051	P=0.954	**P=0.022**	P=0.224
Step Away*	**P=0.007**	**P<0.001**	**P=0.046**	**P<0.001**	P=0.930

Table 3.2 Summary of univariate results for evoked behaviour with * denotes occurrence analysis.

3.3.1 Modelling strategy and interpretation of statistical results

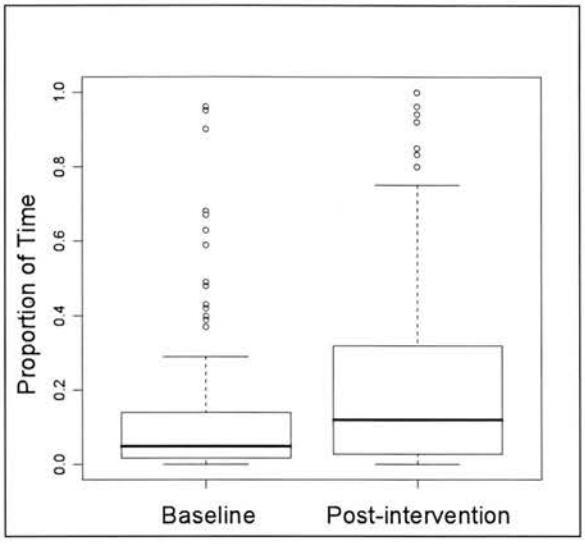
Behaviours indicative of pain are identified by a significant difference between groups after treatment and a significant interaction between the two (section 3.3.1). In this case, an interaction suggests that the change in behaviour associated with treatment is significantly different between groups. The behaviour 'head level' can be used as an example of how this modelling strategy works. The exploratory analysis suggests that time spent with 'head level' increases post-intervention in castrate but not control horses (figure 3.0 and table 3.0). The results of the statistical analysis find a significant difference between groups i.e. between castrate and control horses. This analysis includes both baseline and post-intervention values and can be demonstrated by the box plot in figure 3.1. In this figure it is clear to see that overall all time points castrate horses spend more time with their head level to their withers than control animals.



Behaviour	Group	Treatment	Time	Interaction Group*Treatment	Interaction Group*Time
Head Level	**P=0.008**	**P<0.001**	P=0.184	**P<0.001**	P=0.973

Figure 3.1 Box plot of proportion of time spent with head level in castrated and control horses. Bottom and top of the boxes represent 25 and 75 percentiles respectively with the middle thick black line showing the median value. 'Whiskers' show the interquartile range (adjusted for binomial data). ° shows outliers. The table below shows the results of statistical analysis for this behaviour, with the circled area highlighting the significant effect of experimental group.

The results of univariate statistical analysis also show a significant effect of treatment or baseline compared to post-intervention. This relationship can be seen in figure 3.2. As previously, the analysis compares all baseline data (from both castrate and control horses) to all post-intervention data.

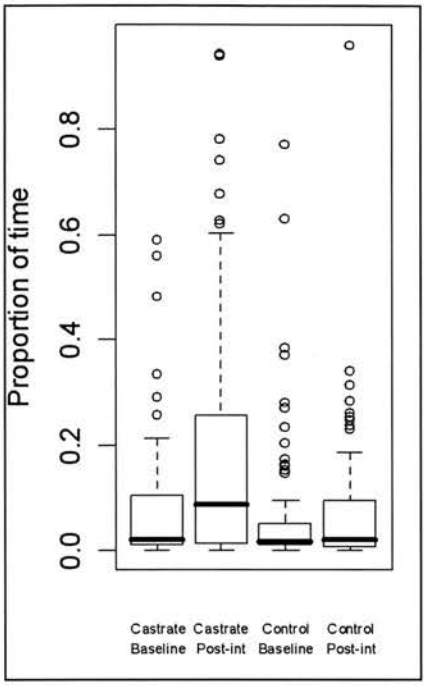


Behaviour	Group	Treatment	Time	Interaction Group*Treatment	Interaction Group*Time
Head Level	**P=0.008**	**P<0.001**	P=0.184	**P<0.001**	P=0.973

Figure 3.2 Box plot of proportion of time spent with head level at post-intervention (castration or sham castration) and baseline periods. Bottom and top of the boxes represent 25 and 75 percentiles respectively with the middle thick black line showing the median value. 'Whiskers' show the interquartile range (adjusted for binomial data). ° shows outliers. The table below shows the results of statistical analysis for this behaviour, with the circled area highlighting the significant effect of treatment.

The statistical analysis performed also identified a significant interaction between group and treatment. Figures 3.1 and 3.2 show that castrate horses spend more time with their 'head level' than control horses and that time spent with 'head level' was greater post-intervention compared to baseline. The interaction between these two parameters indicates that the effect of treatment is not the same in each group i.e. castrate horses respond to treatment in a different way to control horses. Figure 3.3 shows the interaction between group and treatment for 'head level'. The box plot shows that time spent with 'head level' increases in castrated animals following surgery (post-

intervention) but the same level of increase is not seen in control animals following sedation. The interaction between these parameters therefore indicates a behavioural change occurring in association with post-surgical pain.



Behaviour	Group	Treatment	Time	Interaction Group*Treatment	Interaction Group*Time
Head Level	**P=0.008**	**P<0.001**	P=0.184	**P<0.001**	P=0.973

Figure 3.3 Box plot of proportion of time spent with head level in castrate and control horses at post-intervention (castration or sham castration) and equivalent baseline times. Bottom and top of the boxes represent 25 and 75 percentiles respectively with the middle thick black line showing the median value. 'Whiskers' show the interquartile range (adjusted for binomial data). ° shows outliers. The table below shows the results of statistical analysis for this behaviour, with the circled area highlighting the significant interaction between group and treatment.

3.3.1 Behaviours Indicative of Pain

3.3.1.1 Spontaneous Behaviour

Head position was described by three components, head up, down and level with withers. Head low was a grouped behaviour formed by the addition of head down and level.

	Castration	Control
Baseline	0.17 ± 0.2	0.14 ± 0.19
Post-intervention	0.36 ± 0.29	0.2 ± 0.22
Change in behaviour	0.2 ± 0.34	0.05 ± 2.8

Table 3.3 Mean ± standard deviation of proportion of time spent with head low in castrate and control horses at baseline and post-intervention periods and the within group change in behaviour from baseline to post-intervention periods.

Proportion of time spent with head low was significantly affected by group and treatment and a significant interaction between group and treatment ($P<0.009$) indicated that the posture head low was adopted more often post-operatively after castration as can be seen in figure 3.1. Descriptive analysis presented in table 3.3 showed that on average, time spent with head low increased by 20% from baseline to post-intervention in the group undergoing castration. In the control population, receiving only sedation time spent with head low also increased but to a lesser extent (5% on average). There was no effect of time or interaction between group and time ($P>0.111$).

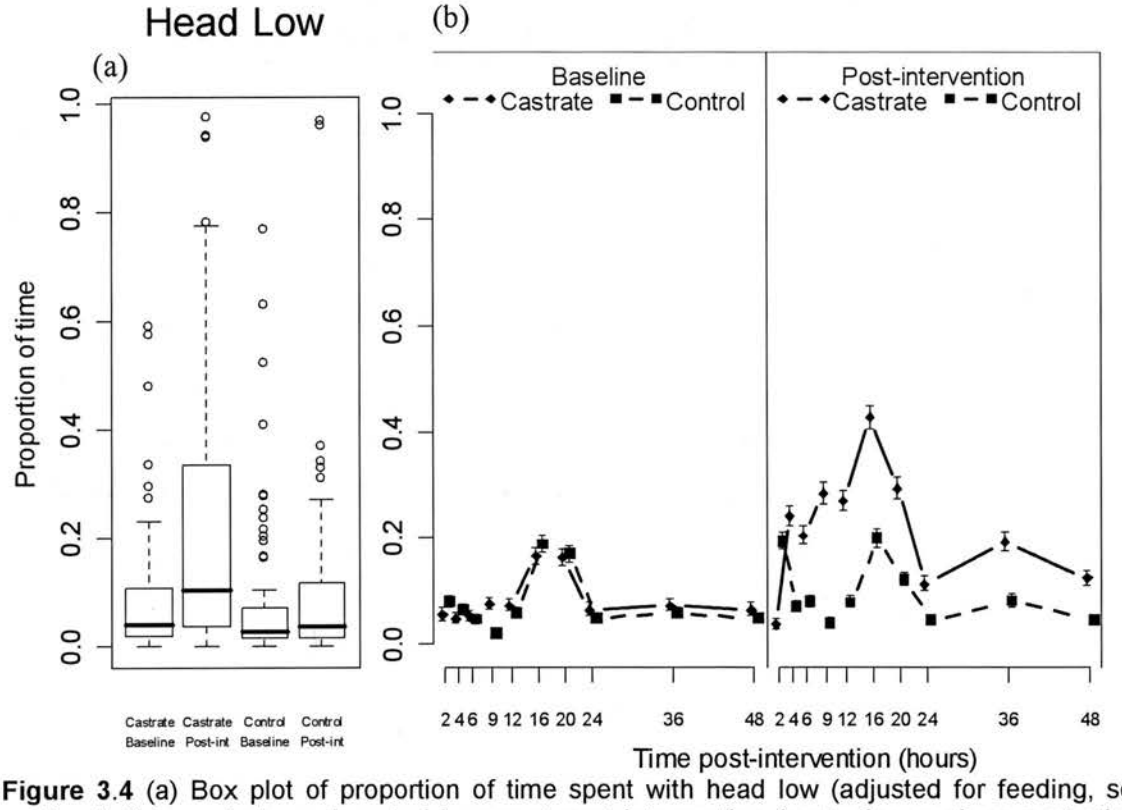


Figure 3.4 (a) Box plot of proportion of time spent with head low (adjusted for feeding, see section 2.2) in castrate and control horses at post-intervention (castration or sham castration) and equivalent baseline times. Bottom and top of the boxes represent 25 and 75 percentiles respectively with the middle thick black line showing the median value. 'Whiskers' show the interquartile range (adjusted for binomial data). ° shows outliers. (b) Line plot showing mean proportion of time (±SE) spent with head low (adjusted for feeding) in castrate (diamond, solid line) and control (square, dotted line) horses at each post-intervention sample point and at equivalent baseline sample points.

Further investigation found time spent with head level to be significantly greater in castrates compared to controls with a significant treatment effect and interaction between group and treatment ($P<0.008$). Proportion of time spent with head down was significantly increased post-intervention ($P<0.001$). However, this change occurred in both groups in a similar manner ($P>0.476$), suggesting it is not due to surgical intervention. Conversely, proportion of time spent with head up (above withers) was significantly reduced during the post-intervention observations in castrate horses ($P<0.01$). There was no effect of time or interaction between group and time ($P<0.111$).

Time point analysis identified a number of changes in head position. Firstly, comparing castrate and control horses identified a significant increase in head level position in castrates at 16 hours and a significant decrease in head up at 20 hours ($P<0.013$). Furthermore, castrate horses showed decreased time with their head up at 6 hours in comparison to pre-intervention baselines ($P=0.014$). Comparing pre- and post-intervention behaviour in control horses found an increase in time spent with head up at 6 hours and a decrease in head level position at 16 hours ($P<0.032$).

Whilst general analysis found no effect, time point analysis identified changes in position in the stable and recumbency that may be considered indicators of pain. Castrate horses spent more time in the middle of the box at 6 hours post-intervention and less time at the front at 16 hours ($P<0.026$) compared to controls. No other differences in box position were identified with time point analysis ($P>0.108$). Castrate horses showed increase lateral recumbency at 16 hours post-intervention compared to controls ($P=0.034$). Sternal recumbency was reduced in control horses at 16 hours compared to pre-intervention control data ($P=0.042$).

3.3.1.2 Evoked Behaviour

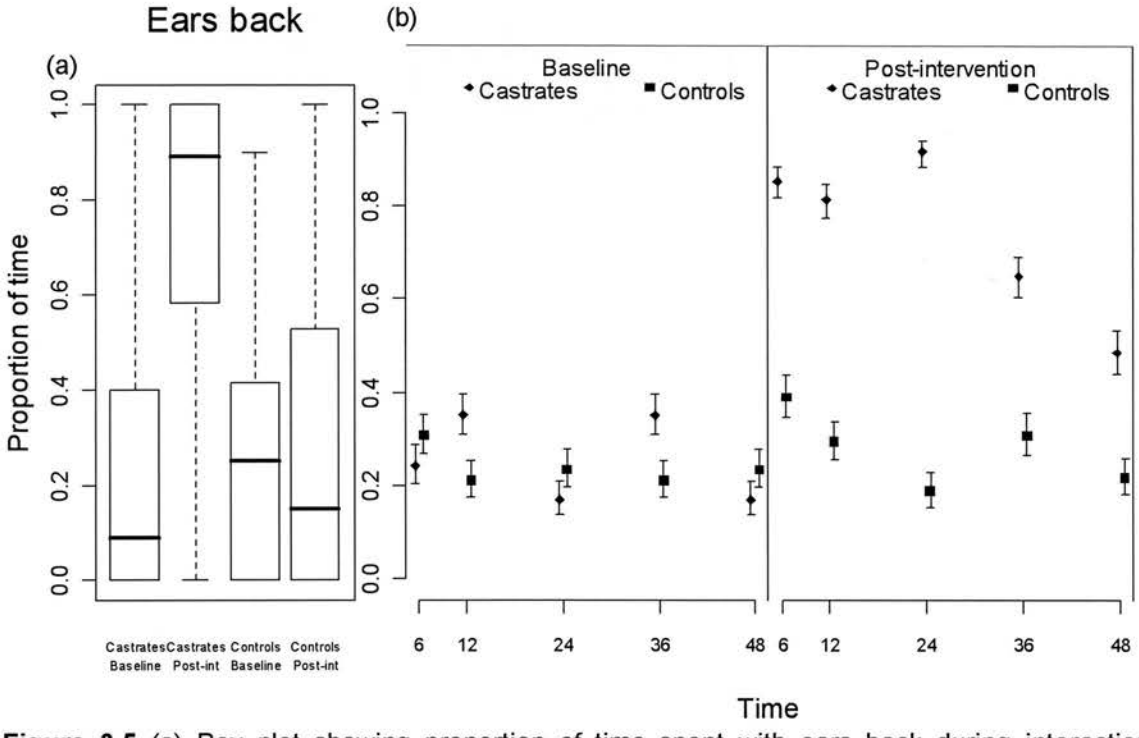


Figure 3.5 (a) Box plot showing proportion of time spent with ears back during interaction testing in castrate and control horses at post-intervention and equivalent baseline times (see figure 3.4). (b) Scatter plot showing mean proportion of time (±SE) spent with head low (adjusted for feeding) in castrate (diamond, solid line) and control (square, dotted line) horses at each post-intervention sample point and at equivalent baseline sample points. Points represent response to a specific test and therefore are not continuous as in Figure 3.1 (b).

	Castration	Control
Baseline	0.26 ± 0.31	0.27 ± 0.28
Post-intervention	0.74 ± 0.33	0.3 ± 0.34
Change in behaviour	0.49 ± 0.42	0.0 ± 0.39

Table 3.4 Mean ± standard deviation of proportion of time spent with ears back during interactive testing in castrate and control horses at baseline and post-intervention periods and the within group change in behaviour from baseline to post-intervention periods.

The proportion of time spent with ears positioned backwards during interactive testing was significantly greater in castrate compared to control horses (P=0.011). There was a significant effect of treatment and interaction between group and treatment (P<0.001), suggesting that change in behaviour with treatment was due to surgical intervention and not sedation. These results are seen in figure 3.5 (a) as an overall increase in 'castrate post-int' and in (b) as elevated values for castrated animals at all time points in comparison with, not only control horses following intervention but pre-intervention (baseline) values for both castrate and control groups. The average increase in time

spent with ears back when compared to baseline values was nearly 50% of the observation period (see Table 3.4). Whilst figure 3.5 (b) suggests a slight decrease in behaviour with time in castrate horses, there was no significant effect or interaction between group and time (P>0.467).

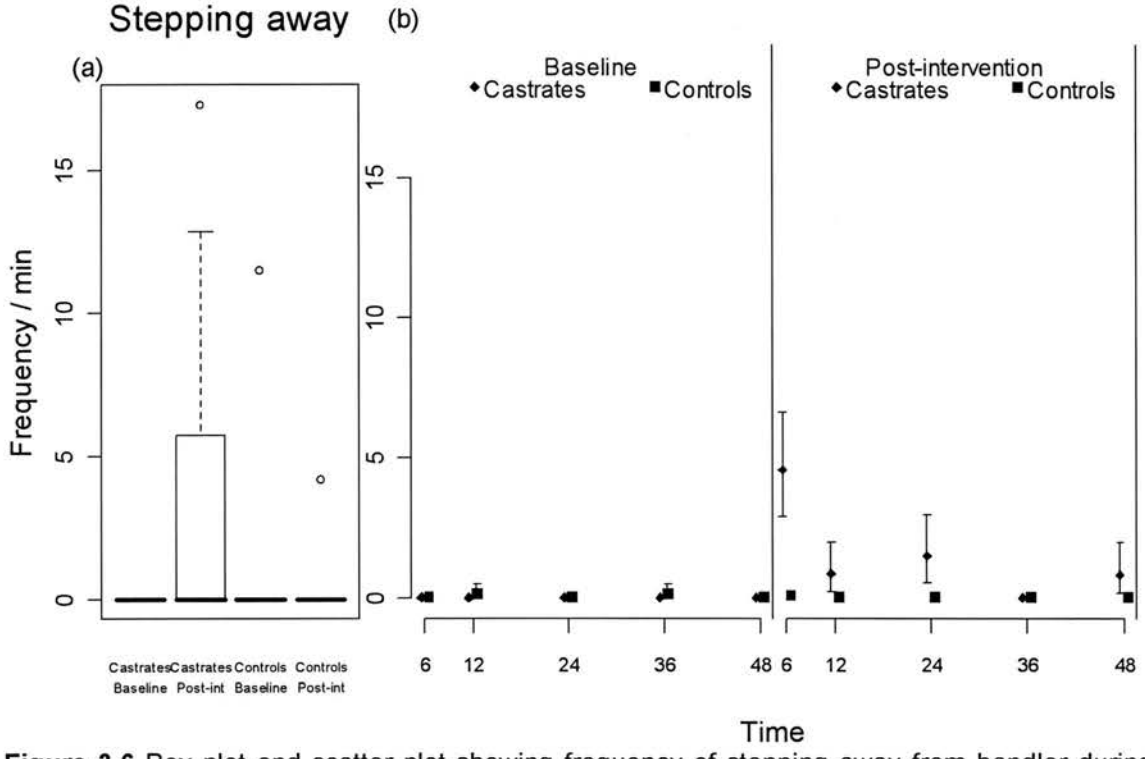


Figure 3.6 Box plot and scatter plot showing frequency of stepping away from handler during interactive testing in castrate and control horses at each post-intervention sample point and at equivalent baseline sample points (see figure 3.1 and 3.2).

Occurrence of stepping away during interactive testing was significantly influenced by experimental group and treatment, with a significant interaction between the two (P<0.007). Figure 3.6 (a) clearly shows an increase in this behaviour in castrate 'post-int'. Occurrence of stepping away pre-intervention is low in both groups, as can be seen in figure 3.6 (b). Post-intervention data show an increased response in castrates at all time points other than 36 hours, with no change in response in control horses. There were no significant interactions between group and treatment or group and time (P>0.930).

3.3.2 Behavioural Effects of Sedation

3.3.2.1 Spontaneous Behaviour

As reported in table 3.1, a number of spontaneous behaviours were shown to be significantly influenced by treatment i.e. behaviour was different before and after intervention ($P<0.049$). However, no significant interaction between group and treatment suggests that changes in behaviour are due to sedation and not pain. Drug effects seen here included an increase in inattentive behaviour, grooming, time spent with head down and time spent at the front of the box ($P<0.049$). Figure 3.7(a) shows this effect, using time spent at the front of the box as an example, with an increase in both castrate and control groups from baseline to 'post-int' observations. Analysis also found an increase in the level of occurrence of stamping and level of occurrence of hindlimb lifting ($P<0.003$) in both groups, indicating an association with sedation.

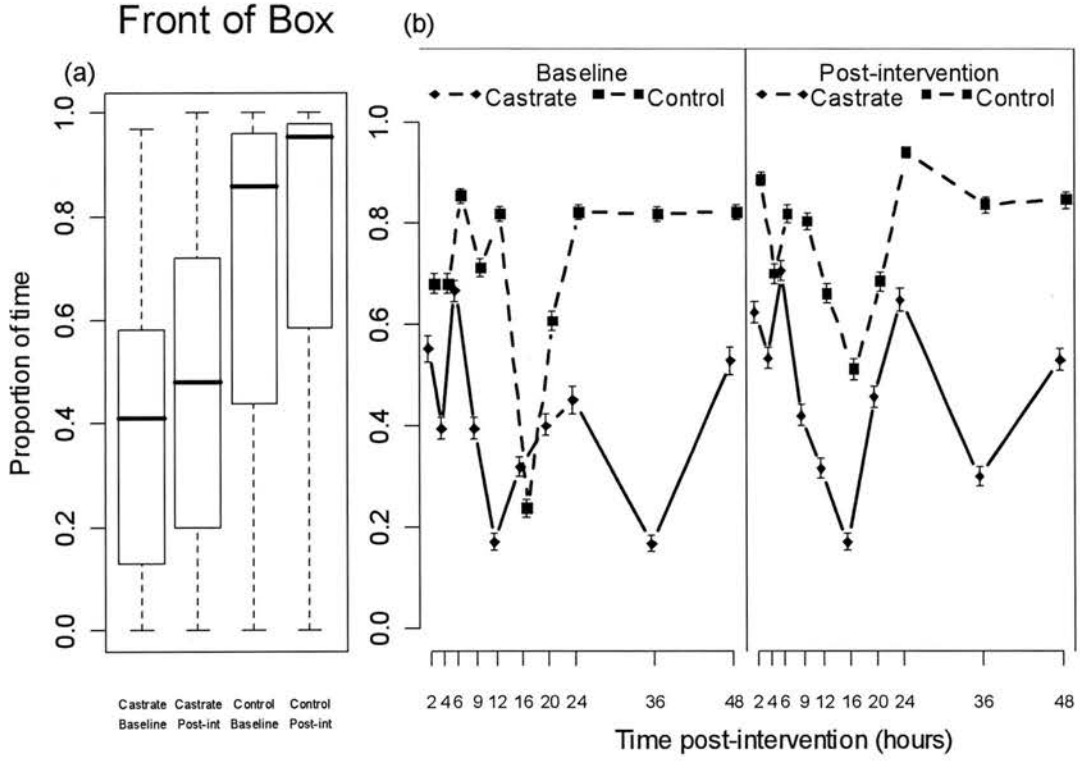


Figure 3.7 Proportion of time spent at the front of the box in castrate and control horses at pre- and post-intervention time points (see figure 3.1).

3.3.2.2 Evoked Behaviour

Evoked behaviour was not affected by sedation, with no significant effects of treatment ($P>0.051$) where a significant group/treatment interaction was not occurring, suggesting

that post-intervention changes were different in castrate and control horses, therefore not attributable to the effects of sedation.

3.3.3 Experimental Group Effects

3.3.3.1 Spontaneous Behaviour

As identified in table 3.1, exploratory behaviour and stable position data varied with experimental group both before and after castration/sham castration. For example, castrate animals spent significantly less time at the front of the box than controls at both baseline and post-intervention time points ($P<0.001$). This suggests a consistent difference between control and castrate animals, an example of which can be seen in figure 3.7a with an overall (pre-and post-intervention) reduced time spent at the front of the box in castrated compared to control animals.

3.3.3.2 Evoked Behaviour

As in section 3.3.2.2, evoked behaviour was not affected by experimental group, with both groups showing similar responses at baseline time points.

3.3.4 Multivariate Analyses

3.3.4.1 Tree-model analyses

Tree-model analysis creates binary splits in the data, which best separate, the experimental groups. The analysis was used here to illustrate possible important behaviours in the assessment of painful and pain-free animals. Univariate general analysis identified differences between experimental groups in time spent exploring and at the middle or back of the stable (see Appendix 2.2). These changes were seen both prior and post-intervention and appear to represent fundamental differences between the experimental groups. Feeding behaviours were also removed from analysis due to unavoidable differences in feeding regimes. Drinking was included as all animals had access to *Ad Libitum* water.

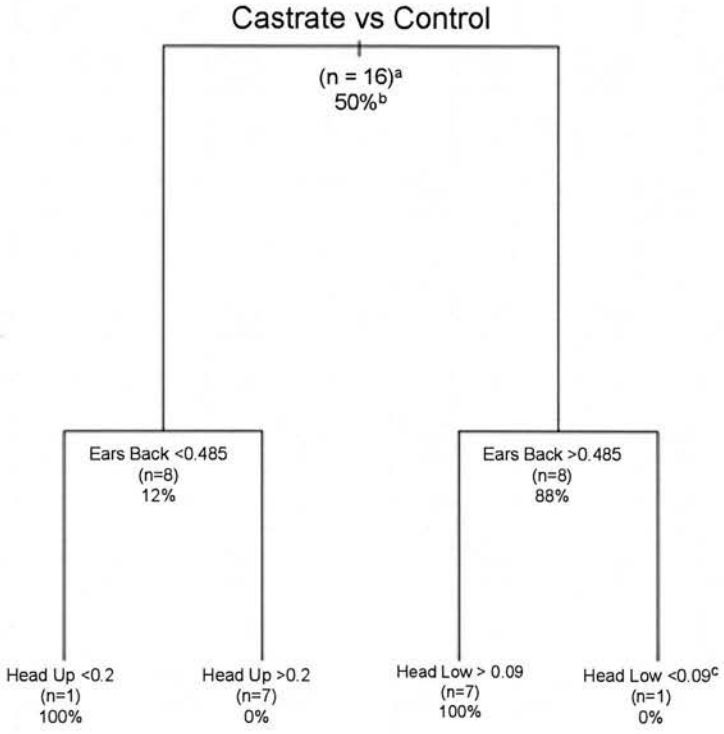


Figure 3.8 Tree-model for identification of castrate and control horses at 12 hours post-intervention. Where value (a) represents the total number of horses in the group, (b) represents the percentage of castrate animals in that group and (c) represents the proportion of time spent (state behaviours) or frequency (event behaviours) of behaviour. For example, at branch 'ears back <0.485' there are 8 horses fulfilling this criteria (n=8), 12% of these are from the castrated group. This diagram shows ears back forming the primary division and head up and head low forming secondary divisions.

Tree-models were generated at 6, 12, 24, 36 and 48 hour post-intervention time points, in order to incorporate both spontaneous and evoked behaviour. Figure 3.8 shows a representative example of the tree diagram generated for 12 hours post-operative, with primary and secondary divisions.

Behaviour	**Division**			
	Primary	**Secondary**	**Tertiary**	**Quaternary**
Forelimb lifting	0	1	0	0
Weight shifting	0	0	1	1
Head up	1	2	0	1
Stepping away (evoked)	1	0	0	0
Ears back (evoked)	2	0	0	0
Head low	0	1	0	0
Head down, turn towards handler (evoked)	1	0	0	0
Standing	0	0	1	1
Head down	0	1	0	0
Head level	0	0	1	0

Table 3.5 Behaviours identified in tree-based models as forming the primary, secondary and tertiary tree 'branches'. Examples of primary and secondary branches can be seen in figure 3.5.

Table 3.5 shows the combined results for all tree diagrams, describing which behaviours formed the primary, secondary etc divisions and the number of times a specific behaviour formed a division. The tree analysis revealed that the evoked behaviour 'ears back' to demonstrated the clearest division, forming the initial branch in 2 out of 5 trees (see figure 3.5), with 88% of castrates performing the behaviour more than 48% of the time at 12 hours and 100% performing the behaviour for less than 69% of the time at 24 hours. Stepping away provided the clearest division at 6 hours and time spent with head down and turned towards the handler during interactive testing formed the preliminary division at 36 hours. Head position was also of importance, forming the initial split at 48 hours post-intervention and secondary splits at 3 out of 5 time points.

3.3.4.2 Discriminant Analysis

As with tree-based models, behaviours which showed significant differences between experimental groups in general analyses (i.e. exploring and time at middle or back of stable) were removed from the dataset. Feeding behaviours were also removed from analysis due to unavoidable differences in feeding regimes. Discriminant analysis was used to simplify the assessment protocol by assessing how accurately different combinations of behaviours allocated horses into their treatment groups.

At 6 hours post-intervention, discriminant analysis determined stepping away during interactive sampling in combination with positioning ears back during an interaction to be the optimal combination of behaviours. Using these parameters, 77.8% of castrate and 100% of control horses were allocated to their corrected groups, resulting in an overall accuracy of 88.9%. If both parameters were removed from the analysis, and 'active response' was used as the single discriminating variable, the accuracy was reduced to 77.8% (castrate: 66.7%, control: 88.9%) using 'active response' as the single discriminating variable. Further removal of 'active response' did not result in a reduction of accuracy, with hind limb lifting alone correctly assigning 77.8% of horses to their correct group (castrate: 66.7%, control: 88.9%).

At 16 hours post-intervention a combination of 'ears back' during interaction, 'head level' and 'hindlimb weight-shifting' allocated 100% of horses to their correct group. Following removal of these parameters, discrimination was not possible.

3.4 DISCUSSION

Changes in equine behaviour suggestive of post-operative pain or discomfort were found in both spontaneous and evoked behaviour. Importantly for the accuracy of any further clinical pain assessment protocols, a number of behaviours altering in association with sedation were identified, which should be corrected for in future studies. The following tables summarise the results for univariate and multivariate analysis. Subsequently, important findings are discussed in more detail.

3.4.1 Summary of results

Results of univariate general analysis found behaviour was significantly affected by one or more fixed effects such as experimental group or treatment. The combination of significant results for experimental group, treatment and interaction between group and treatment indicated that there was a significant treatment effect, which was different between groups and was potentially indicative of pain. Significant results for 'treatment' suggest a post-intervention change in both groups, considered to be indicative of the effects of sedation. Behaviours indicative of pain and sedation are summarised in table 3.6 and 3.7.

Pain		Sedation	
↓	Head up	↑	Inattentive
↑	Head level	↑	Grooming
↑	Head low	↑	Head down
↑	Ears back during interaction	↑	Front of box
↑	Stepping away during interaction	↓	Weight shifting
		↑	Stamping
		↑	Hindlimb lifting

Table 3.6 Behaviours increased (↑) or decreased (↓) in the presence of pain and sedation

Multivariate analysis using tree-models identified which behaviours formed the best division between post-intervention castrate and control horses, hence between 'pain-free' and 'painful' animals. This technique was used to indicate the importance of particular parameters. It is generally accepted that accurate identification of pain may not rely solely on one parameter, with multifactorial assessment protocols improving accuracy and reliability. Discriminant analysis determined which behavioural parameter

or combination of parameters most accurately assigned horses to their appropriate groups i.e. painful castrate and pain-free control.

Tree-based Models	Discriminant Analysis	
	6 hours	16 hours
Ears back (evoked)	Stepping (evoked) + ears back (evoked)	Ears back (evoked)+ head level + hindlimb weight shifting
Head Position	Active response (evoked)	
Stepping away (evoked)	Hindlimb lifting	
Head down and turned towards the handler (evoked)		

Table 3.7 Behaviours identified as important in the discrimination between painful (castrate) and 'pain-free' (control) horses.

Unsurprisingly, both univariate and multivariate analyses have highlighted similar key behaviours, with an accuracy of discrimination of 88.9% at 6 hours post-intervention and 100% accuracy at 16 hours.

3.4.2 Behaviours Indicative of Pain

3.4.2.1 Head Position (spontaneous behaviour)

Results suggest head position to be a possible indicator of acute post-surgical pain in the horse. Univariate analysis highlighted that, over a 48 hour period, castrated horses spent significantly more time with their head low (level with or lower than the withers) than sedated control horses post-intervention. Horses consequently spent significantly less time with their head up. Non-themed grouped behaviours, such as 'head low', were incorporated in the assessment protocol in situations where similar behavioural elements that may be easily confused, leading to inaccuracies i.e. head down (below withers) and head level (level with withers). Splitting 'head low' behaviour into its components found that time spent with head down was increased in both castrate and control horses post-intervention but time spent with head level was only increased in castrate horses. This suggests that increased 'head down' position may be representative of sedative effects, whereas head level may be indicative of pain. The use of grouped behaviours, may simplify possible assessment protocols. However, in the case of head position, it appears that whether the head is level with or below the withers represents different states and therefore it is important to differentiate between the two postures.

In a preliminary study examining castration under standing surgical anaesthesia in horses, Eager (2002) found an increase in time spent with head level at 6 hours post-operative in comparison to non-sedated controls. Price et al. (2003) also found differences in head position when comparing post-surgical arthroscopy patients with non-anaesthetised controls. In this chapter time spent with head above withers was found to be greater in control horses compared to post-recovery arthroscopy patients. Positioning the head level with withers is characteristic of a resting posture in the horse (McDonnell 2003) and therefore may reflect increased post-operative resting in castrate horses. Pritchett et al. (2003) also found increased resting in horses (defined as '*no movement or activity while standing with attention to the environment or in a restful posture*' pg. 35) following exploratory celiotomy. Increases in resting behaviour have been noted in other species including pigs (Taylor et al. 2001) and in chickens following painful intervention (Gentle et al. 1999; Hocking et al. 2001; Hocking et al. 2005). Post-surgical resting may result from reluctance to move and pain minimisation or conservation of resources to promote quick recovery. However, in the current study the themed group 'inattentive' was composed of seven behaviours (resting hindlimb, head level or down, no oral behaviour, no stereotypical behaviour, standing at the middle or back of the stable) thought to indicate a resting posture. However, inattentive behaviour was found to be altered by sedation but not pain specifically. This may result from the addition of the conflicting behaviours head level (pain) and head down (sedation). It is possible that, considering only time spent with head level as a component of inattentive posture my alter results. Generally, a lowered head carriage may reflect lack of interest in or withdrawal from the external environment.

The identification of two very similar behaviours with different implications highlights the need for detailed, objective research into pain behaviour, rather than reliance on subjective assumption. For instance, Raekallio et al. (1997a, 1997b) scored head position as part of a multifactorial pain assessment protocol. In these studies, head above withers was given a score of '0', head level with withers was given a score of '1' and head below withers '2'. Where scales and scoring systems are based on previously validated indices, such as in Thornton and Waterman-Pearson (1999), results may not be affected and ease of monitoring improved. However, Raekallio et al. (1997b) found

poor correlation between the parameters measured in their study, possibly due to fundamental failings in the assessment protocol.

3.4.2.2 Stepping away (evoked behaviour)

The use of palpation or interaction tests to facilitate assessment of animal pain through the provocation of a more noticeable behavioural response (Morton & Griffiths 1985; Sanford et al. 1986) has been recommended in situations of mild pain (Rutherford 2002). Following a surgical procedure, tissue damage and inflammation may result in hyperalgesia and allodynia (see section 1.1), increasing pain experienced in mildly painful situations and eliciting painful responses to non-noxious stimuli. The interactive test employed in the current study aimed to generate a tactile stimulus, which would not normally be perceived as painful or threatening. In the presence of noxious stimuli the horse will attempt to escape or if this is not possible, become aggressive (Casey 1999). Stepping away during an interactive test may represent escape attempts, with the post-operative increase in this behaviour seen in the current study and that of Clarke (2001), therefore suggesting hypersensitivity to a previously innocuous interaction.

3.4.2.3 Ears back (evoked)

Increased time spent with ears backwards during interactive testing identified here confirmed the results of a preliminary study (Eager 2002). Pinning or flattening the ears backwards has been associated with aggressive interactions in the horse (McDonnell 2003), offering protection to the ears (Kiley-Worthington 1997; Goodwin 2002). The increase in proportion of the interactive test spent with ears back in this study may represent an aggressive 'warning' in a situation where the horse is fearful.

3.4.5 Behaviours Indicative of Sedation

A number of spontaneous behaviours were found to change post-intervention in both castrate and control horses. An increase in 'inattentive' posture was seen post-intervention possibly suggesting increased resting. α_2 agonist sedation results in a clinically recognised posture (Bryant et al. 1991), inactive, with legs spread and head down (as seen in figure 3.9). Whilst the expected duration of action of detomidine should not exceed two hours (see section 2.3.1.1), the results of this study suggest that subtle behavioural effects extend longer than previously identified. Position in the box has been marked as a potential indicator of equine pain (Price et al. 2003), although results of the study reported in this chapter were not significant. In the current study, time spent at the front of the box was also affected by sedation, decreasing post-intervention. Time spent grooming was reduced post-intervention in both groups, contrasting to results of a previous study were grooming was decreased in castrate but not control horses (Eager 2002). However, control horses in this study had not been sedated and therefore the drug effects would not have been taken into account. Further research is needed to elucidate effects of alternate sedative and anaesthetic protocols, including general anaesthesia, on behaviour, in order to confirm findings of the study reported here.

Figure 3.9 Posture characteristic of sedation with an α2 agonist.

3.4.6 Effects of Experimental Group

The current study highlighted a number of behaviours which were significantly different between groups pre-intervention. Whilst there was no statistically significant difference in age between the two groups, control horses were, on average, older than castrated animals. This may have slighted altered behaviour with older animals having more experience of different surroundings and have been better handled. In addition, control horses were all geldings. Lower levels of circulating testosterone (Voith 1979) may have some effect on aggressive and reactive behaviour. However, it should be considered that these differences may reflect the large individual variation in behaviour

seen here. The use of mixed effects modelling for general analysis not only examined the effect of treatment and experimental group but also the interaction between these parameters. The simultaneous between- and within-group analysis identified the magnitude of treatment-based changes in each group and determined whether or not there was a significant difference in the nature of behavioural change in response to treatment. In this manner, the effects of treatment could be examined, independent to the experimental group effects.

3.4.7 Types of Analyses

A number of analysis techniques have been employed in the current study, each with separate aims. Differences in time point analysis, tree-based model and discriminant analysis results at different time points may reflect varying levels of drug activity, difference pain phases (i.e. initial tissue insult graduating to inflammatory pain) or effects of large individual variation in a small sample size. Financial and time constraints have limited the sample size used in the current study which may have limited the efficacy of multivariate techniques and hindered the identification of changes in less frequently occurring behaviours. However, in general analyses have identified similar behaviours namely head position, the evoked behaviours stepping away and ears back and with some suggestion that position in the box and recumbency may also be of use.

3.5 CONCLUSION

In conclusion, the current study has identified behavioural parameters which are indicative of pain or discomfort in the horse, including spontaneous behaviours such as head position and hindlimb movements and evoked behaviours such as stepping away and positioning ears backwards during an interactive test. The results suggest that evoked behaviour may be of more use than spontaneous behaviour in the assessment of acute post-surgical pain. There is a general lack of consensus within the veterinary community regarding the severity of pain associated with procedures such as castration (Price et al. 2002), with some veterinarians discounting the presence of pain entirely (Green 2001). The results of the current study may, therefore, reflect the possible mild severity of the procedure used or the effectiveness of the analgesic agents provided.

The study reported here used 'in the field' castration under standing surgical anaesthesia as a model for acute post-surgical pain. However, castration and many other surgical procedures are also carried out under general anaesthesia in a hospital situation (Price et al. 2005), where the accuracy of behavioural pain assessment protocols may be heavily influenced by the lasting effects of general anaesthesia and unfamiliar environments (Taylor et al. 2002b). The following chapter examines the effects of these factors on the equine castration model.

CHAPTER FOUR

OBJECTIVE ASSESSMENT OF BEHAVIOURAL RESPONSES TO CASTRATION AND SHAM CASTRATION UNDER GENERAL ANAESTHESIA

4.1 INTRODUCTION

Optimal pain management is fundamentally reliant on the ability to accurately identify and assess pain in animals. Pain can lead, not only to intense suffering, but also to prolonged hospitalisation, impaired wound healing and increased morbidity and mortality (Otto & Short 1998; Flecknell 2000). Behaviour is perhaps the most accessible tool for the assessment of post-operative pain (Hansen 1997), with changes occurring immediately (Anil et al. 2002). Objective and subjective behavioural observations have been used for the assessment of pain severity in horses (Raekallio et al. 1997a; Raekallio et al. 1997b; Price et al. 2003; Pritchett et al. 2003), however, in order to produce a valid, accurate and efficient tool for equine pain assessment further work is need. The work described in the previous chapter (chapter three) aimed to identify behavioural indicators of acute post-surgical pain through the examination of responses to castration performed under standing surgical sedation in the animal's home environment. However, for many equine surgical procedures, patients are admitted to a hospital and undergo general anaesthesia, both of which may result in further behaviour changes, confounding behavioural pain assessment protocols.

The behavioural effects of general anaesthesia in horses have yet to be clarified and until this has been achieved, effective pain assessment and in turn pain management, in the post-operative period will be hindered. Pritchett et al. (2003) used a group of horses anaesthetised for non-surgical purposes for comparison with animals undergoing exploratory celiotomy. Horses undergoing general anaesthesia without surgery were more active post-intervention than control horses (hospitalised without intervention), predominantly in the first hour, due to increased feeding motivation following post-anaesthesic starvation. Comparing pre-and post-anaesthetic behaviour in clinically pain-free horses, Seibert et al. (2003) suggested anaesthesia to result in decreased time with

the ears up and forward and increased hindlimb resting for up to 20 hours. No changes in head position were noted.

Castration of horses under general anaesthesia is commonly performed 'in the field' or in a hospital setting (Price et al. 2005). This method is frequently used as a preferred method of restraint for difficult cases or where patients are too small to allow the procedure to be performed standing (Mason et al. 2005). Castration under general anaesthesia is also a necessity in the more mature horse or cryptorchid animals. This general anaesthesia castration model (see section 2.1) therefore, not only aims to examine pain responses to a commonly performed surgical procedure but will also elucidate any effects of 'anaesthetic hangover' potentially enabling more effective pain assessment (and therefore management) in more generalised situations, including when the horse is hospitalised. The null hypotheses specifically addressed in the current study were;

6) Behaviour will not be altered in association with castration pain.
7) Behaviour will remain consistent over time
8) There will be no statistically significant behavioural effects of general anaesthesia.
9) There will be no significant difference between times of maximal and minimal analgesia.
10) Behavioural parameters will not accurately discriminate between castrated and control horses.

4.2 METHODOLOGY

4.2.1 Subjects

Ten clinically normal, TB/warm blood colts (age: 16 months ± 9 (mean ± SD)) were admitted to the Royal (Dick) School of Veterinary Studies (R(D)SVS) for elective castration under general anaesthesia. Informed client consent was obtained prior to participation in the study. In addition, ten clinically normal, TB/warm blood control horses (age: 41 months ± 18 (mean ± SD)) were admitted to the Large Animal Hospital of the R(D)SVS, treated in the same manner as castrate horses and underwent sham castration (see section 4.2.4). Details of all horses can be found in Appendix 3.1. General anaesthesia of control horses was carried out under Home Office licence and ethical approval was granted from the University of Edinburgh Ethical Review Committee for this project.

4.2.2 Management

All horses were admitted to the hospital and allowed a minimum of 12 hours for habituation to surroundings prior to commencement of 24 hours of baseline observations. Horses were starved from midnight on the night prior to surgery. For clarification, figure 4.0 shows the experimental timeline. All horses were maintained in loose boxes (4 m^2 x $5m^2$) with shavings over a concrete floor surface. Water was available *Ad Libitum* and haylage was provided, as is normal hospital procedure.

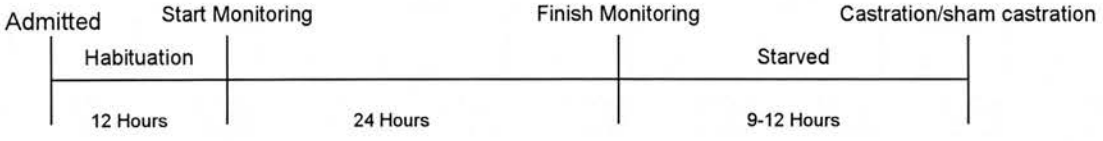


Figure 4.0 Pre-intervention timeline for castrate and control horses.

4.2.3 Anaesthesia and Analgesic Protocol

All horses were weighed on an equine weigh-bridge (castrate: 366 ± 59 kg, control: 474 ± 92 kg (mean ± SD)). On entry to the induction box, horses were pre-medicated with xylazine (1.1 mg kg^{-1}) via IV catheter and flunixin meglumate (1.1 mg kg^{-1} IV) was administered. Anaesthesia was induced five minutes later with intravenous injection of

ketamine (2.2 mg kg^{-1}) and diazepam (25 μg kg^{-1}) in combination. Following endotracheal intubation, horses were transferred to the operating theatre.

Inhalational anaesthesia was maintained on a circle breathing system (Large Animal Control Centre, Draeger Medical, Herfordshire, U.K.) with halothane in oxygen (50%) and nitrous oxide (50%). End tidal halothane concentration was maintained at 0.8 – 1.0%. Nitrous oxide was discontinued after 10 minutes.

Ketamine and midazolam were administered by bolus injection to effect, if anaesthetic 'top up' were deemed necessary. Hypotension (MAP <58mmHg, < 5 minutes) was controlled by infusion of dobutamine (0.5 μg kg^{-1} min^{-1}) until MAP increased to 68mmHg. Acepromazine (0.01 mg kg^{-1} IV) was administered in the case of hypertension (MAP<108 mmHg, persistent for > 5 minutes).

Morphine (0.12 mg kg^{-1} IV) was administered during surgery. Post-operatively, phenylbutazone (2.2 mg kg^{-1}) was given twice daily, PO.

4.2.4 Surgical Procedure

Following induction of general anaesthesia, horses were moved into the operating theatre and positioned in dorsal recumbency. In castrated horses, routine aseptic procedures were performed and closed castration performed by one of two experienced soft tissue surgeons. Control horses were maintained under general anaesthesia in dorsal recumbency for 1 hour (sham castration). The duration of the procedure was not longer than 60 minutes.

4.2.5 Assessment Protocol

Assessment of both castrate and control horses included undisturbed spontaneous behavioural monitoring and evoked behavioural assessment, details of which can be found in section 2.2. Collection of baseline data was commenced 12 hours following hospitalisation, in order to allow horses' time to habituate to new surroundings. Baseline data were obtained over a 24 hour period prior to pre-intervention starvation. Time '0' was established as the point at which the horse returned from the recovery box

to its original stable. Detailed observations and evoked behavioural tests were performed at, and point samples of undisturbed spontaneous behaviour were taken from video tapes at 2, 4, 6, 9, 12, 16, 20, 24, 36 and 48 hours post-intervention and at equivalent baseline times i.e. if 2 hours post-operative fell at 1400h a baseline sample was taken at the same time of day.

4.2.6 Statistical Analysis

Statistical analysis was performed as in chapter three. Detailed explanations of all statistical techniques can be found in section 2.5. Time point analysis was performed at 6 and 16 hours post-intervention, representing states of minimal and maximal analgesia respectively (see section 2.3.3.2).

4.4 RESULTS

Control horse 10 was removed from analysis due to post-anaesthetic colic. As in section 3.3, preliminary exploratory analysis examined the data graphically to identify potential behavioural variables indicative of pain and to recognise any effects of variables other than pain. Figure 4.1 shows a selection of the preliminary screening graphs.

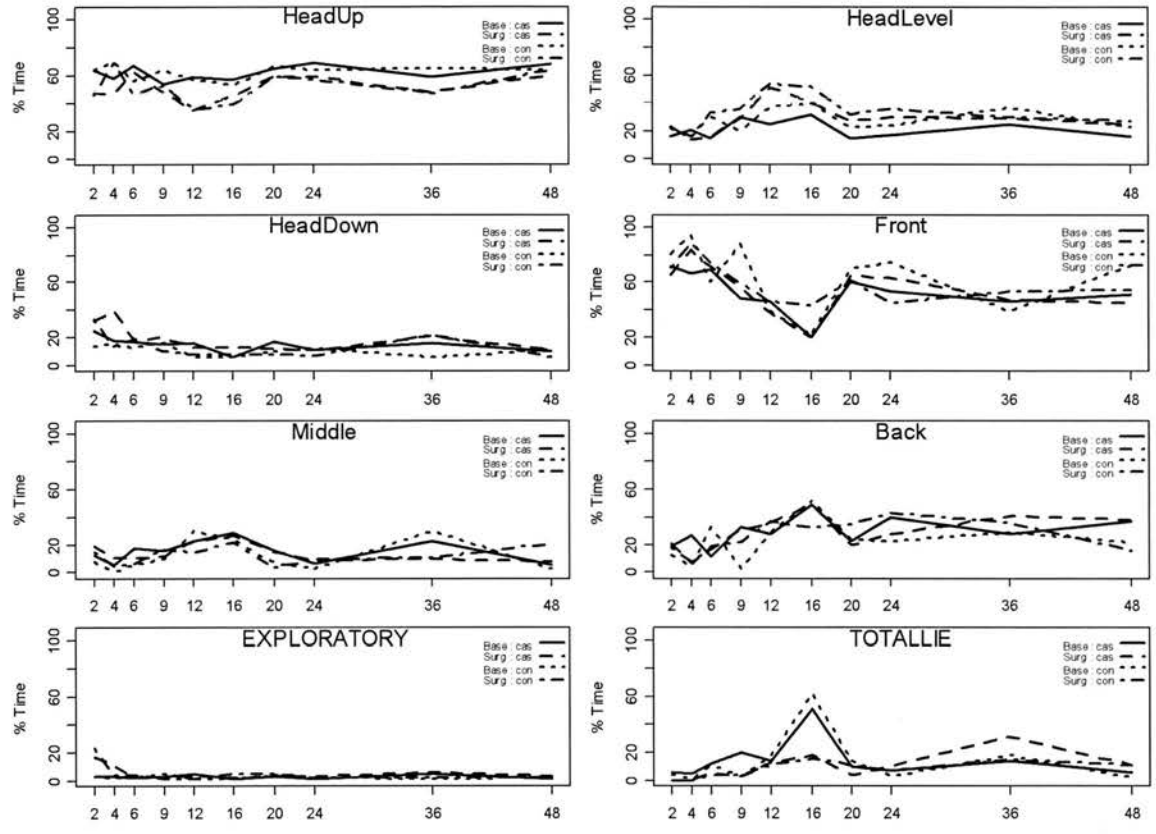


Figure 4.1 Preliminary graphical analysis of individual behavioural variables showing proportion of time spent performing each behaviour by castrated and control groups at baseline and post-intervention periods.

Examination of the screening graphs could find no potentially indicators of pain. As can be seen in figure 4.1 when all four lines fall closely together there is no clear pattern and it is difficult to assess whether or not any experimental effects are occurring.

Table 4.0 shows a summary of significant results for univariate general analysis of spontaneous (including detailed observation behaviours) behaviours and table 4.1 shows results of evoked behavioural assessment. Complete tables of results (including degrees of freedom, test statistics etc) can be found in Appendix 3.2 – 3.5.

Behaviour	Group	Treatment	Time	Interaction Group*Treatment	Interaction Group*Time
Inattentive	P=0.308	**P<0.001**	P=0.326	P=0.338	P=0.776
Total lying	P=0.508	**P=0.039**	**P=0.028**	P=0.875	P=0.939
Head low	P=0.955	**P<0.001**	P=0.063	P=0.659	P=0.403
Exploratory	P=0.564	**P=0.002**	**P=0.013**	P=0.978	P=0.351
Stand	**P=0.008**	P=0.212	**P=0.002**	P=0.623	P=0.559
Rest hindlimb	**P=0.002**	**P<0.001**	P=0.107	P=0.679	P=0.636
Lie sternally	P=0.244	**P=0.028**	**P=0.041**	P=0.543	P=0.946
Lie laterally	P=0.487	P=0.358	P=0.090	**P=0.037**	P=0.611
Grooming	**P=0.026**	P=0.417	P=0.616	P=0.260	P=0.106
Head up	P=0.927	**P<0.001**	**P=0.042**	P=0.840	P=0.616
Head down	**P=0.018**	**P=0.023**	**P<0.001**	P=0.843	P=0.591
Head Level	P=0.085	**P<0.001**	P=0.431	P=0.752	P=0.968
Front	P=0.236	P=0.727	**P<0.001**	P=0.298	P=0.811
Back	P=0.463	P=0.621	**P<0.001**	P=0.401	P=0.439
Hindlimb move	P=0.842	**P=0.002**	**P=0.038**	P=0.172	P=0.398
Weight Shift	P=0.636	**P<0.001**	P=0.695	P=0.290	P=0.488
Lift Forelimb	P=0.856	**P=0.003**	P=0.064	P=0.273	P=0.363
Lift Hindlimb	P=0.860	P=0.462	**P=0.016**	P=0.567	P=0.641
Stamp*	P=0.066	**P<0.001**	P=0.558	P=0.999	**P=0.05**
Tail Flick*	P=0.223	P=0.677	P=0.590	**P=0.030**	P=0.743
Tail Flick≠	P=0.421	P=0.484	**P=0.009**	P=0.087	**P<0.001**
Head Shake≠	P=0.280	P=0.138	**P=0.006**	**P=0.01**	P=0.639
Ears forward	P=0.747	**P<0.001**	P=0.582	P=0.122	P=0.209
Ears side	**P=0.005**	**P<0.001**	P=0.046	P=0.450	P=0.730
Hindlimb lift*	P=0.096	P=0.394	**P=0.043**	**P<0.001**	P=0.805
Tail flick*	**P=0.005**	P=0.196	P=0.272	P=0.740	P=0.005
Skin twitch*	**P=0.018**	P=0.053	P=0.196	**P=0.016**	P=0.184
Stamp*	**P=0.032**	P=0.066	P=0.055	P=0.641	P=0.345

Table 4.0 Summary of univariate results for spontaneous behaviour. * denotes occurrence analysis and ≠ level of occurrence analysis.

Behaviour	Group	Treatment	Time	Interaction Group*Treatment	Interaction Group*Time
Ears back	**P<0.001**	**P<0.001**	P=0.467	**P<0.001**	P=0.730
Head up (turn towards)	P=0.493	**P=0.011**	P=0.578	P=0.667	P=0.960
Head down (straight)	P=0.435	**P<0.001**	P=0.587	P=0.999	**P=0.025**
Exploratory	P=0.131	**P<0.001**	P=0.242	P=0.062	P=0.983
Stamp*	P=0.999	**P<0.001**	P=0.111	**P<0.001**	P=0.999
Step Away*	**P=0.007**	**P<0.001**	P=0.320	**P=0.017**	P=0.369
Tail Flick*	P=0.178	P=0.848	P=0.250	**P<0.001**	P=0.446
Ear Flick*	P=0.999	P=0.111	**P<0.001**	P=0.999	**P<0.001**

Table 4.1 Summary of univariate results for evoked behaviour. * denotes occurrence analysis and ≠ level of occurrence analysis.

4.3.1 Behaviours Indicative of Pain

As previously described in chapter three, behaviours which may be indicative of pain are indicated by a significant difference in group and treatment and a significant interaction between the two. For further discussion, with an example, please see section 3.3.1.

4.3.1.1 Spontaneous Behaviour

There were no spontaneous behaviours identified that were indicative of pain, i.e. where a significant effect of treatment and experimental group, in addition to a significant interaction between treatment and group, was found.

4.3.1.2 Evoked Behaviour

4.3.1.2.1 Ears back

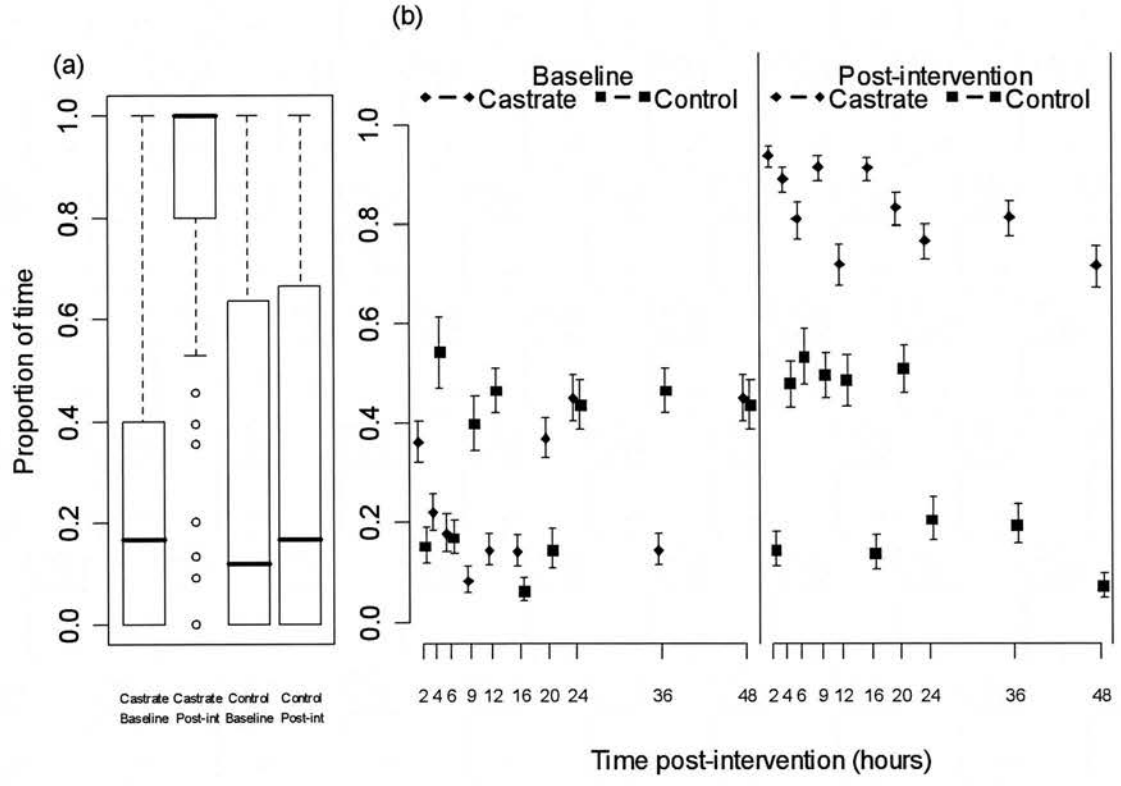


Figure 4.2 Box plot and scatter plot showing proportion of time spent with ears back during interactive testing in castrate and control horses at each post-intervention sample point and at equivalent baseline sample points (see figure 3.1 and 3.2 for full description of the plot).

Time spent with ears back during evoked behavioural test was significantly different in castrate compared to control horses ($P<0.001$). There was a significant effect of treatment and a significant interaction between group and treatment ($P<0.001$). The data shown in table 4.2 suggest a 58% increase in the time spent with 'ears back' during interaction in castrated horses after surgery with a limited change in behaviour following sedation in control horses. This increase is clear in the above box plot (figure 4.2a). There was no significant effect of time or interaction between group and time ($P>0.467$).

	Castration	Control
Baseline	0.26 ± 0.29	0.31 ± 0.36
Post-intervention	0.84 ± 0.27	0.33 ± 0.38
Change in behaviour	0.58 ± 0.4	-0.02 ± 0.49

Table 4.2 Mean ± standard deviation of proportion of time spent with ears back during interactive testing in castrate and control horses at baseline and post-intervention periods and the within group change in behaviour from baseline to post-intervention periods.

4.3.1.2.2 Stepping away

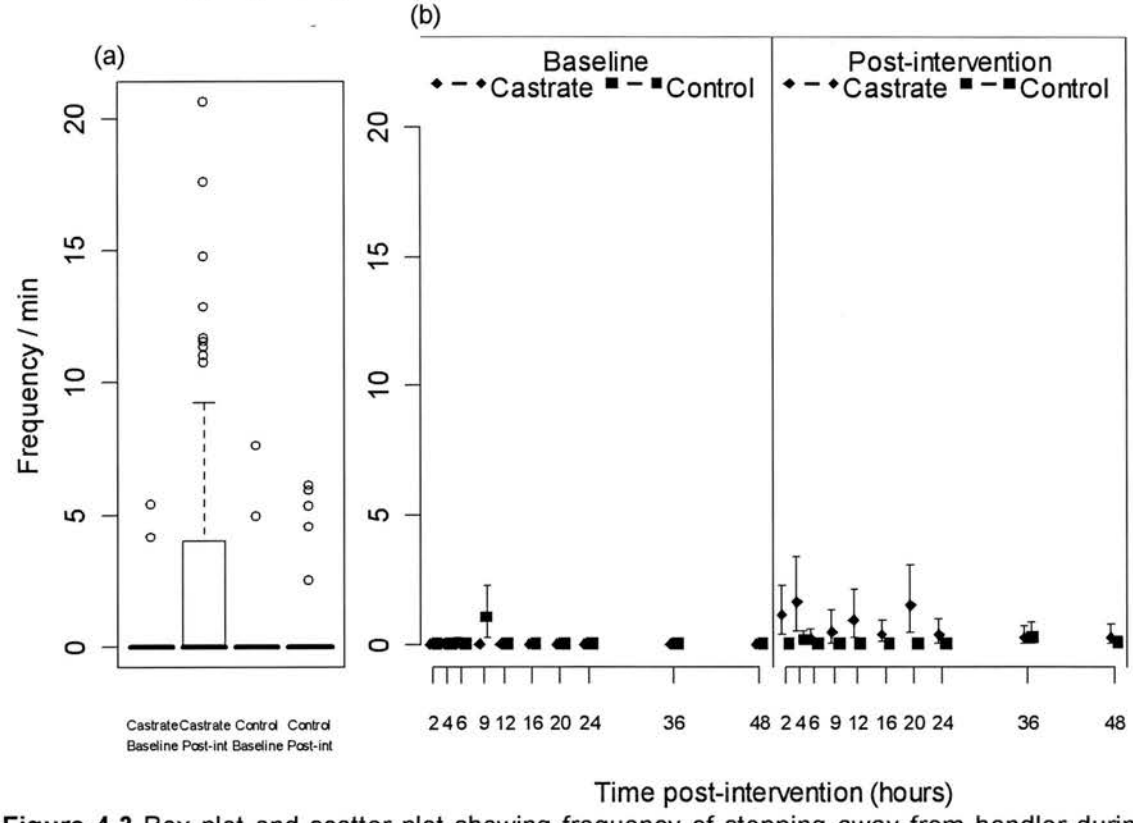


Figure 4.3 Box plot and scatter plot showing frequency of stepping away from handler during interactive testing in castrate and control horses at each post-intervention sample point and at equivalent baseline sample points (see figure 3.1 and 3.2).

Square root transformation of stepping away data was not sufficient to obtain a normal distribution of residuals due to high numbers of zero values. Occurrence analysis found a significant effect of group and treatment on frequency of stepping away during interactive testing and a significant interaction between treatment and group ($P<0.007$) as can be seen in figure 4.3(a) by the increased post-intervention values in the castrate but not control group. There was no effect of time and no interaction between group and time ($P>0.320$). Level of occurrence was not affected by group, treatment or time and there was no interaction between group and treatment or group and time ($P>0.095$).

4.3.2 Behavioural Effects of General Anaesthesia

4.3.2.1 Spontaneous Behaviour

Table 4.1 identifies behaviours that show a significant result for treatment ($P<0.039$) but not group or interaction between group and treatment ($P>0.066$). The lack of significant group effects and a significant interaction suggests that changes in behaviour are occurring following intervention in both groups, therefore being indicative of anaesthetic effects rather than pain. As an example, figure 4.4 shows the effect of treatment on time spent with head low, with both box and line plots showing clearly increased post-intervention values. There was no significant effect of experimental group or time ($P>0.063$, figure 4.4b). A significant difference between baseline and post-intervention values ($P<0.001$) was seen, however, there was no interaction between experimental group and treatment ($P=0.403$, figure 4.4a).

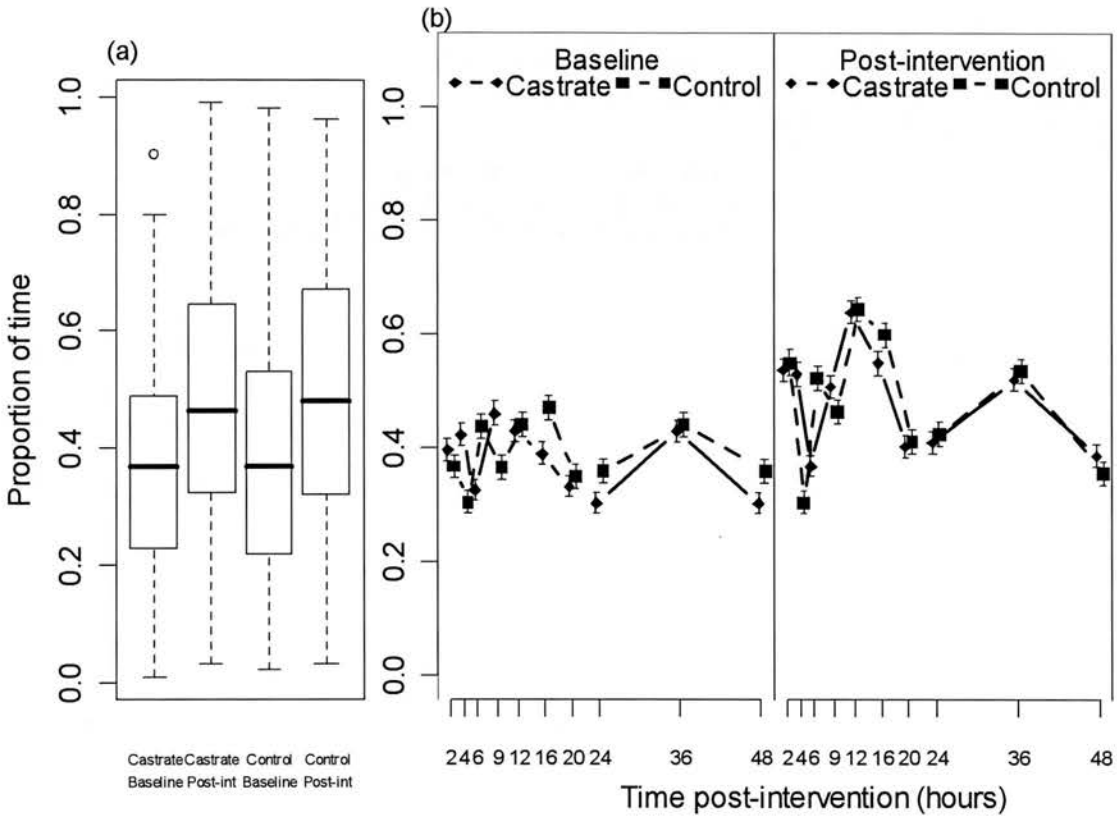


Figure 4.4 (a) Box plot of overall proportion (±SE) of time spent with head low (adjusted for feeding) in castrate and control horses at post-intervention (castration or sham castration) and equivalent baseline times. (b) Line plot showing mean proportion of time (±SE) spent with head low (adjusted for feeding) in castrate (diamond, solid line) and control (square, dotted line) horses at each post-intervention sample point and at equivalent baseline sample points.

Figure 4.4 shows a representation of anaesthesia effects. Further effects of anaesthesia can be seen as an increase in inattentive behaviour, hindlimb resting, exploratory behaviour, ears sideward and leg movements (Table 4.1). Behaviours reduced in association with anaesthesia include recumbency, head up and ears forward.

4.3.2.2 Evoked Behaviour

Similar treatment effects were also found in evoked behaviour. Behavioural changes occurred post-intervention, to the same extent in both castrated and control groups and are therefore potentially associated with general anaesthesia. These changes include decreased time spent with head up and turned towards the handler and exploring the handler and increased time spent with head down and straight and occurrence of stamping ($P<0.011$).

4.3.3 Experimental Group Effects

4.3.3.1 Spontaneous Behaviour

4.3.3.1.1 Resting a Hindlimb

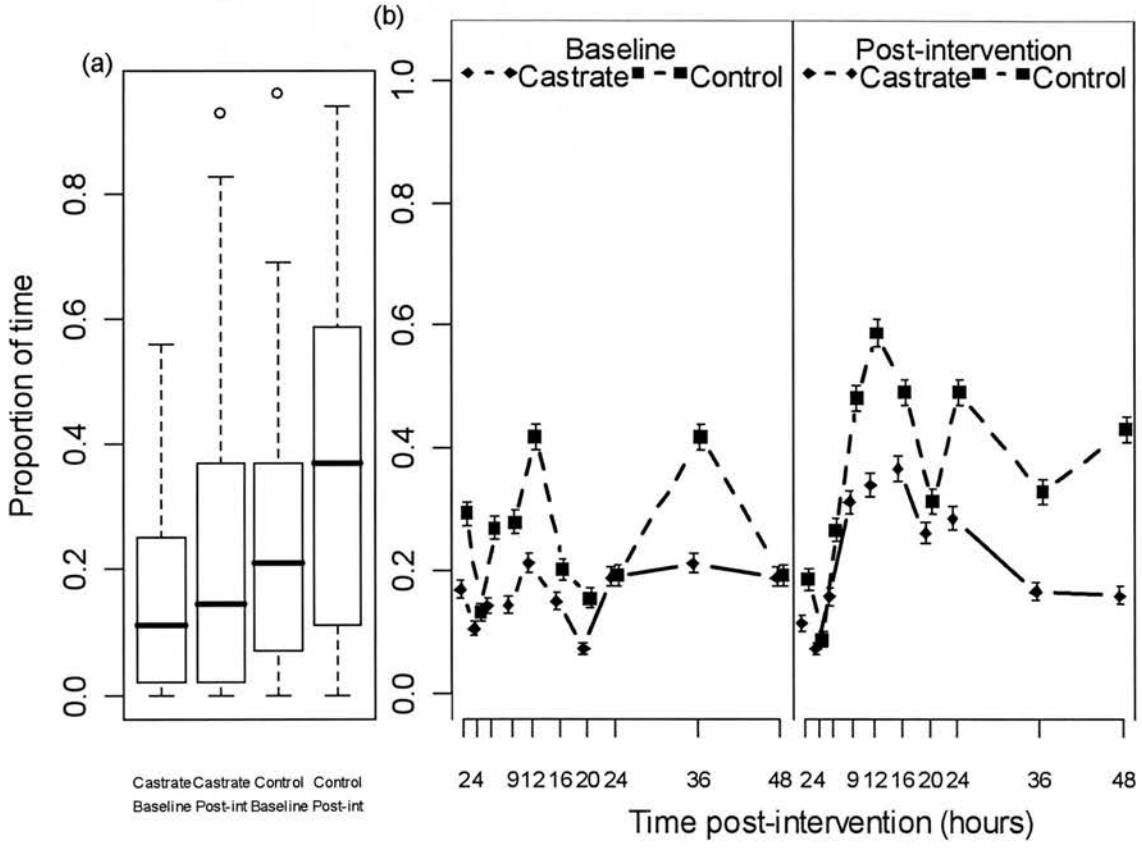


Figure 4.5 Proportion of time spent in the resting a hindlimb in castrate and control horses at post-intervention and equivalent baseline time points (see figure 4.1).

Proportion of time spent resting a hindlimb was significantly greater in control compared to castrate horses and post-intervention compared to baseline ($P<0.002$). There was, however, no interaction between experimental group and treatment, effect of time or group:time interaction ($P>0.107$). Behaviours showing a similar response included proportion of time spent standing, grooming, head down, ears sideway and occurrence of tail flicking, skin twitching and stamping.

4.3.3.2 Evoked Behaviour

In contrast to spontaneous behaviour, no effects of experimental group alone were seen in evoked behaviours ($P>0.131$), i.e. there was no significant difference in the way castrate and control horses reacted to the interactive test.

4.3.4 Tree-model Analysis

Tree-based models were drawn for each post-intervention time point (i.e. 2, 4, 6, 9, 12, 16, 20, 24, 36, 48 hours). As in chapter three, analysis was used here to illustrate potentially important behaviours in the assessment of pain in horses, through the comparison of all trees and examination of occurrence frequency of each behaviour at primary, secondary and tertiary divisions. Behaviours showing significant pre-intervention differences between groups (see section 4.3.3) were removed from analysis. A representative tree diagram (figure 4.6) has been included showing ears back forming the primary division and walking forming the secondary division.

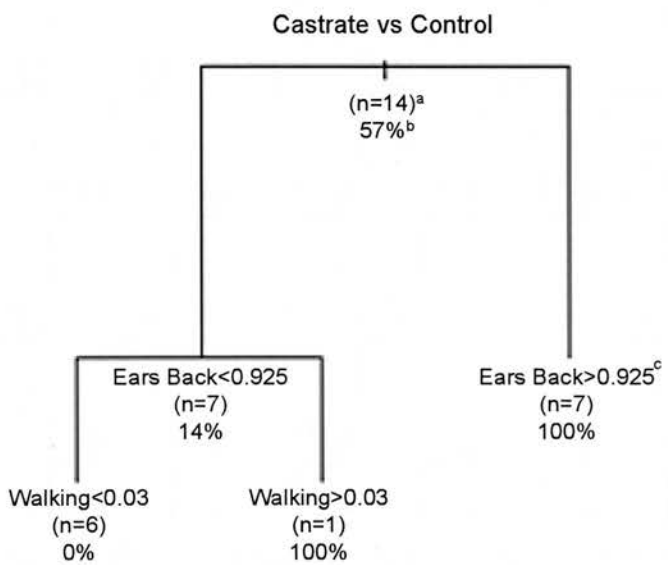


Figure 4.6 Tree-model for identification of castrate and control horses at 16 hours post-intervention. Where value (a) represents the total number of horses in the group, (b) represents the percentage of castrate animals in that group and (c) represents the proportion of time spent (state behaviours) or frequency (event behaviours) of behaviour. This diagram shows ears back forming the primary division and walking forming the secondary division.

Table 4.3 is a summary of all tree-based model results, showing behaviours forming primary, secondary and tertiary branches and the number of times these divisions were created.

Behaviour	Division		
	Primary	Secondary	Tertiary
Ears back (evoked)	7	0	0
Head level	1	0	1
Back of box	1	1	0
Walking	1	2	1
Lateral recumbency	0	1	0
Hind limb lifting	0	1	0
Head up and straight (evoked)	0	1	0
Front of box	0	1	1
Head up, turned towards handler (evoked)	0	0	1
Forelimb lifting	0	2	0
Head down	0	1	0
Head low	0	1	0
Weight shift	0	1	0

Table 4.3 Behaviours identified in tree-based models as forming the primary, secondary and tertiary tree 'branches'

'Ears back' during interactive testing appeared to be the most important behaviour, forming the clearest (primary) division between experimental groups in 7 out of the 10 trees drawn. Time spent walking formed the primary division in one out of ten trees and appeared as a secondary divider twice and once as a tertiary divider. 'Head level' formed one primary division and one tertiary division.

4.3.5 Discriminant Analysis

Discriminant analysis was performed on data from 6 and 16 hours post-intervention. As in tree-model analysis behaviours which showed significant differences between experimental groups in general analyses were removed from the dataset.

At 6 hours post-intervention, head level alone provided optimum discrimination between groups. This parameter assigns 78.6% of horses to their correct groups (castrate - 88.9%; control – 60%). When 'head level' was removed, it was not possible to distinguish between castrate and control animals on the basis of the behavioural variables remaining.

At 16 hours post-intervention, a combination of (increased) ears back during interactive sampling, (reduced) exploratory behaviour and (increased) head shaking provided 100% discrimination between groups. Following removal of these parameters, discrimination between castrated and control animals was not possible.

4.4 DISCUSSION

4.4.1 Summary of Results

Results of univariate general analysis found spontaneous and evoked behaviour was significantly affected by one or more 'fixed' effects (i.e. treatment, experimental group or time), indicating influences of pain, anaesthetic hangover and experimental group. Behaviours potentially indicative of pain and general anaesthesia are summarised in table 4.4.

Pain		General Anaesthesia	
↑	Ears back during interaction	↑	Head low
↑	Stepping away during interaction	↑	Inattentive behaviour
		↑	Hindlimb resting
		↑	Exploratory behaviour
		↑	Ears sideways
		↑	Leg movements
		↓	Recumbency
		↓	Head up
		↓	Ears forward
		↑	Head up - towards handler (evoked)
		↑	Exploring handler (evoked)
		↑	Head down – straight (evoked)
		↑	Stamping

Table 4.4 Behaviours increased (↑) or decreased (↓) in the presence of pain and general anaesthesia

Further to univariate analysis, multivariate techniques were used to determine the relative importance of different behaviours in the separation of experimental groups and to determine the optimum combination of parameters for optimal discrimination between groups. The results of tree-based model and discriminant analysis are summarised in table 4.5, showing behaviours highlighted as important by these techniques.

Tree-based Models	Discriminant Analysis	
	6 hours	16 hours
Ears back (evoked) Head level Back of box Walking Lateral recumbency Hindlimb lifting Head up and straight (evoked) Front of box Head up, turned towards handler (evoked) Forelimb lifting Head down Head low Hindlimb weight-shift	Head level	Ears back (evoked) + Head shaking

Table 4.5 Behaviours identified as important in the discrimination between painful (castrate) and 'pain-free' (control) horses.

4.4.2 Behaviours Indicative of Pain

4.4.2.1 Stepping Away & Ears Back (Evoked Behaviour)

As in chapter three, both stepping away and ears back during interactive testing were highlighted as potential indicators of pain in univariate and multivariate analysis. Pritchett et al. (2003) formed a 'socialisation' score from the summed response to a number of positive stimuli. Following exploratory celiotomy, surgical patients were less responsive than anaesthetised controls. Positive stimuli included, opening the stable door, offering grain, approaching and lifting the feet. In the current study, reactions of surgical patients suggested an escape/aggressive response, contrary to the reduced reactivity in the previous study. This difference may be due to the nature of the condition studied. Equine colic may be associated with shock and toxaemia as well as severe pain (Taylor et al. 2002b), therefore horses in the 2003 study may therefore be suffering from the effects of illness in addition to pain which may have a modulating effect on their behaviour. Following castration surgery, the horses in the current study may be experiencing acute pain due to tissue injury and inflammation. This may result in pain hypersensitivity, mediating increased protective reactions to non-noxious stimulation. Additionally, the interactive (palpation) test used in the current study elicited a defensive reaction when stimulation was gradually moved towards the surgical site. Pritchett et al. (2003) observed responses to the lifting of the horses feet

following exploratory celiotomy, which has little relevance to the surgical site and therefore may not have been threatening.

4.4.2.2 Head Position

Whilst pain-associated changes in head position were not identified in general univariate analysis, both tree-model and discriminant analysis results suggest time spent with 'head level' may be an important discriminator between castrated and control horses. Moreover, in contrast to results reported here, (Seibert et al. 2003) found no effect of general anaesthesia on head position in clinically normal horses, anaesthetised for 2 hours. Control horses here, undergoing inhalation isoflurane anaesthesia for a period of approximately one hour showed decreased time spent with 'head above the withers'. As previously discussed in section 4, sedation and pain-related changes in head position are easily confused and therefore care is required when determining their inclusion in a pain assessment protocol. These behaviours are very closely linked and could easily be confused. Whether or not the inclusion of these slightly ambiguous behaviours would improve or reduce the accuracy of a multifactorial assessment protocol requires further investigation.

4.4.3 Behaviours Indicative of General Anaesthesia

Pritchett et al. (2003) identified increases in active behaviour immediately post-recovery in horses anaesthetised for non-painful procedures. The current study, however, identified a number of behavioural changes in association with anaesthesia alone (see table 4.4). This has been noted by Seibert et al. (2003) highlighting changes in behaviour following isoflurane anaesthesia. In the current study, time spent resting a hindlimb was found to increase in association with anaesthesia. Likewise, an increase in post-anaesthetic hindlimb resting in clinically normal horses was also reported by Seibert et al. (2003) confirming this anaesthetic-associated change in behaviour.

Previous studies show increased exploratory or active behaviour subsequent to recovery from anaesthesia in both surgical (Price et al. 2003) and horses anaesthetised for non-painful procedures (Pritchett et al. 2003). This change in behaviour was considered most likely to be representative of increased feeding motivation and foraging. Decreased

post-operative activity after celiotomy (Pritchett et al. 2003), but not after castration/arthroscopy may reflect differences in the severity of surgical insult and pain..

4.4.4 Effects of Experimental Group

As in chapter three, pre-intervention differences in behaviour were seen between groups. This may have related to differences in age and perhaps may be due to the fact that control horses had been previously gelded. Equine castration is performed in order to reduce undesirable 'masculine' behaviour such as mounting (Houpt 1999), although some geldings retain 'studish' behaviours (Voith 1979). Consequently, the geldings used in the control group may show differences in aggressive and reactive behaviours compared to pre-castration colts. However, as described in chapter three, the use of generalised mixed effects models for statistical analysis and the incorporation of 'baseline' data allowed each group to act 'as its own control' permitting the examination of differences in *'change in behaviour'* (pre- compared to post-operative) between experimental groups.

4.5 CONCLUSION

Univariate analysis failed to identify statistically significant differences in spontaneous behaviour, however, statistically significant differences in evoked behaviour were seen between horses that were castrated under general anaesthesia and those that received general anaesthesia alone. Tree-based analysis and discriminant analysis identified ears back, but not stepping away as potentially important indicators. In addition, multivariate analysis identified behaviours such as time spent with 'head level to withers' which potentially may be useful in combination with other behaviours, included in a multifactorial assessment protocol. The results, therefore, suggest that evoked behaviours may be more easily and quickly identifiable indicators of post-operative pain. However, as results of discriminant analysis suggest, careful inclusion of spontaneous behaviours may improve accuracy. It is also important that the anaesthesia-associated behavioural changes identified are taken into account when formulating assessment protocols and evaluating analgesic treatments.

Chapters three and four have examined behavioural responses to an acute post-surgical pain state, in two similar clinical situations. Whilst the improved management of acute pain in these situations is an obvious concern for veterinary clinicians, as management can improve wound healing and reduce hospitalisation times. However, the recognition, assessment and management of chronic pain is equally difficult and represents a major concern. Indicators of pain may be specific to acute or chronic pain (Anil et al. 2002) and it should not be assumed that behavioural changes are consistent between pain states. Chapter five examines behavioural adaptations seen in association with chronic digital pain in laminitic horses in order to find pain state specific and general pain associated behaviours.

CHAPTER FIVE

OBJECTIVE CHARACTERISATION OF BEHAVIOURAL INDICATORS OF LAMINITIC PAIN IN THE HORSE

5.1 INTRODUCTION

Laminitis is a systemic disease, characterised by the dysadhesion of the distal phalanx and inner hoof wall lamellae (Pollitt 1999; Hood 1999a). Disruption of the dermal and epidermal laminar bond results in structural weakness within the hoof capsule which can lead to rotation and sinking of the pedal bone, often leading to extreme, unmanageable pain (Hood 1999a). Laminitis is a chronic, debilitating, yet common condition, therefore representing a huge welfare concern. Treatment is extremely difficult as clinical signs only become apparent once a number of pathological changes have already occurred (Bailey 2004). In a study of 113, 000 U.K. equines Hinckley and Henderson (1996) identified a prevalence of 7.1%, indicating a major welfare concern to the equine industry.

Laminitis can be classified into four distinct phases of disease; *developmental, acute, subacute* and *chronic* laminitis (Hood 1999a). The developmental phase describes the period following the causative insult and the appearance of lameness, which can be up to 60 hours. Unfortunately, whilst many irreversible pathological changes occur in this period, the developmental phase is relatively asymptomatic (Pollitt et al. 2003). Acute laminitis describes the first appearance of lameness and clinical signs of laminitis. If the horse remains in this phase for more than 72 hours without evidence of digital collapse, the phase is then described as sub-acute. Digital collapse is identified as the rotation or sinking of the 3rd phalanx, following which the horse is described as a chronic laminitic (Hood 1999a).

Clinical signs of laminitis include lameness of varying severities; a pounding digital pulse; warming of the feet; reluctance to move and weight-shifting between feet (Swanson 1999; Hood 1999a) Further to this, laminitic horses may develop a characteristic stance, with weight shifted back on to the heels and hind feet positioned

under the belly, representing an effort to increase load on the heel in preference to the toe area (Swanson 1999).

At present the causal factors and pathogenesis of the disease are poorly understood, with a number of mechanistic hypotheses proposed. It is thought that excessive consumption of certain types of carbohydrate trigger changes in the hindgut that precipitate the disease (Bailey et al. 2004). Additionally, laminitis may be elicited by digital trauma, sepsis and hypovolemia (Hood 1999b). Carbohydrate-induced laminitis is the most common form of disease in the U.K. (Hinckley & Henderson 1996) resulting from accidental starch overload and access to fructan (a storage carbohydrate) rich pasture (Pollitt et al. 2003). The links between factors released from the hindgut into the circulation and the pathogenic processes in the hoof have yet to be elucidated (Bailey 2004). Techniques for the experimental induction of laminitis have included corn starch overload (Garner et al. 1975), insulin administration (French et al. 2000), and oral administration of black walnut extract (Waguespack et al. 2004). Whether or not clinical laminitis follows the same developmental stages as these induced models is still uncertain.

The pathogenesis of laminitis has been explained by two different hypotheses. Establishing whether a specific event occurring during laminitis is a critical step in disease progression or a non-contributing consequence is problematic (Hood 1999b). It is currently unclear as to which hypothesis, if not both in parts, is correct (Bailey 2004). The importance of clinical signs such as increased hoof wall temperature and digital pulse suggested a vascular component to the disease prompting the formation of the vascular hypothesis (Moore et al. 2004). This theory suggests that initial alterations in the digital vascular to be the primary pathology with subsequent problems (i.e. structural weakness and inflammation) occurring as a result of ischaemic necrosis of the epidermal cells (Morgan et al. 2003).

The metabolic or enzymatic hypothesis proposes that causal agents trigger changes in the metabolic processes of the laminar epidermal cells or basement membranes, resulting in dysadhesion of the distal phalanx and inner hoof wall. All further changes are subsequent to structural dysfunction. Study of normal hoof concentrated on the

problem of how the inner hoof wall lamellae remained attached to the stationary distal phalanx whilst during constant hoof wall growth (Pollitt et al. 2003). In the normal hoof, epidermal cells and their basement membranes respond to hoof growth and movement by constant cellular reorganisation (Pollitt et al. 2003). This process is mediated by the release of matrix metalloproteinases (MMPs) and the inhibition by tissue inhibitors of matrix metalloproteinases (TIMPs) (Leach & Oliphant 1983). It is proposed that uncontrolled MMP release may be a mechanism of laminitis (Pollitt et al. 1998) with an increase in the expression of MMP-2 identified following causative insult and previous to the development of first clinical signs (Kyaw-Tanner & Pollitt 2004). Additionally, due to the pathological separation of the basement membrane and lamellar epithelium, reconstruction of the lamellae, as is possible in the normal hoof, is prevented (Pollitt et al. 2003).

Establishment of a acurate prognosis in the laminitic patient is extremely difficult (Herthel & Hood 1999), as it is hard to determine to what extent rehabilitation will be successful. At present, therapy cannot arrest or block the progress of laminitis (Pollitt et al. 2003), and therapeutic goals aim to limit the chances of digital collapse and protect the foot (Hood 1999a). Due to the pathological changes occurring in the developmental and acute phases, once a horse has entered the chronic phase, recovery is rare.

Laminitis is rarely considered a direct cause of death in the horse (Hood 1999a). More commonly patients are euthanased due to inability to adequately manage pain; cost of treatment; long-term inability to perform and poor chances of recovery (Fiester & Mann 2000). Conventional analgesic agents are often inadequate in severe cases, with horses experiencing debilitating foot pain and recumbency (Herthel & Hood 1999; Swanson 1999; Hood 1999a; Pollitt et al. 2003). Chronic pain can be incapacitating and fatiguing (Crane 1987). Restricted movement in these animals may result in reduced food and water intake and restriction of social behaviour. Immune function may be impaired, increasing the probability of viral or bacterial infection (Page 2005). Whether or not equine pain is considered analogous to human pain, chronic laminitic pain is a considerable welfare concern.

Laminitic pain has a number of potential origins including increased concentration of inflammatory mediators; increased submural pressure; traumatic tearing of submural tissue; excessive contact at the solar surface of the distal phalanx; abnormal stress on tendons and ligaments; secondary sepsis, ischaemia and reperfusion injury (Morgan et al. 1999). However, novel findings suggest that the pathological changes occurring during laminitis may result in the generation of a neuropathic pain state (Jones et al. 2007), offering a possible explanation for the ineffectuality of NSAID therapy in severe cases.

Future research developing and assessing novel analgesic agents, targeting inflammatory and neuropathic pain states, is vital. However, without a validated, reliable and reproducible method of pain assessment this cannot be achieved. Traditionally, the Obel grading system (Obel 1948) has been employed by clinicians to monitor and assess laminitic pain. The scale has been modified from the original; the current system is described below in table 5.0;

Score	Description
0	Normal
I	Standing horse lifts feet incessantly; when walking, no lameness is seen; when trotting, the gait is short
II	Horse moves willingly at a walk, but gait is characteristic for laminitis; forefoot can easily be lifted
III	Horse moves reluctantly and resists attempts to lift a forefoot
IV	Horse does not move without being forced

Table 5.0 Obel scoring system grading of lameness associated with laminitis (taken from Morgan et al. (1999)).

In order to reduce interpretative problems a further, clinical grading system (table 5.1) has been developed for the assessment of lameness associated with laminitis (Morgan et al. 1999).

Score	Description
1	Horse is capable of full athletic function
2	Horse is capable of minimum pleasure riding but not full athletic function
3	Horse cannot be ridden but is usable for breeding or can be maintained pasture sound with minimal use of systemic analgesics
4	Horse must be maintained on systemic analgesics to function
5	Horse must be euthanased due to severe unresponsive pain

Table 5.1 Clinical scoring system of lameness associated with laminitis (taken from Morgan et al. (1999)).

These scales have been adopted into clinical practice without validation or clarification of the intra- and inter-observer reliability (Keegan et al. 2003). Whilst including more descriptive terms within a scoring system may improve realiabiltiy, both scales comprise entirely of subjective criteria, for instance, how much 'force' is required for an animal to be 'forced'? The development of the clinical grading system aimed to reduce interpretative problems; however, the performance-related criteria used are ambiguous and easily influenced by observer preconceptions and experience (Keegan et al. 2003). The applicability of the clinical scale for use in the assessment of analgesic agents is confounded by the use of analgesic therapy as a determinant for scoring. The scale, therefore, also represents a somewhat circular argument; providing analgesia where lameness is present, whilst using the provision of analgesia as an indicator of degree of lameness. Both the Obel and clinical grading system provide tools for the monitoring and recording of laminitis-associated lameness, however, these scales are associated with a significant level of inter-observer variability (Viñuela-Fernández et al. 2007) and therefore may not be adequate for the study of novel analgesic therapies.

Additional complications arise from the fact that pain severity may not be directly associated with degree of pathology (Morgan et al. 1999).A number of behavioural adaptations have been described in association with laminitic pain, including weight shifting between feet and the adoption of abnormal standing postures (Swanson 1999). It appears that there is only one study in which objective measurement of laminitic pain associated behaviour has been undertaken, however, the frequency of weight shifting was the sole parameter examined (Rietmann et al. 2004).

The further advancement of knowledge of pain in association with laminitis and the development and assessment of novel pain management strategies relies fundamentally on ability to accurately and objectively assess and monitor pain severity. Therefore, the aim of this study was to identify behavioural indicators of laminitic pain associated with laminitis, for inclusion in an evidence-based assessment protocol. The null hypotheses addressed in this chapter are;

1. There will be no significant difference in behaviour between laminitic and control horses.

2. Behavioural variables measured will not discriminate between laminitic and control horses.
3. No difference in behaviour will be seen in laminitic horses at times of minimum and maximum analgesia.
4. External factors such as time of day will have no influence on equine behaviour.

5.2 METHODOLOGY

5.2.1 Subjects

Seven horses were admitted to the Large Animal Hospital of the Royal (Dick) School of Veterinary Studies for the management of acute laminitis (treatment group). Participation was determined by one experienced clinician based on the presence of clinical signs consistent with the disease. These signs included multi-limb lameness, increased amplitude of the digital pulses, warmth across the dorsal hoof wall and a laminitic gait (Stashak 1987). Informed client consent was sought prior to admission onto the study. Seven clinically normal, age and sex matched horses, which were considered 'pain free' (control group) throughout the duration of the study. Subject details can be found in Appendix 4.1.

5.2.2 Maintenance

Horses were maintained in loose boxes (4m x 5m) with shavings and had free access to water. Laminitic animals were fed restricted rations of soaked hay, whereas control animals all received haylage *Ad Libitum*. Control horses were stabled directly opposite the laminitic horses allowing simultaneous recording to discount any extraneous event in the stable that may have caused alterations in behaviour.

5.2.3 Therapeutic regime

Laminitic horses received phenylbutazone twice daily (2.2 – 4 $mg.kg^{-1}$ IV BID - Equipalazone, Arnolds Veterinary Products, UK) at 0800h and 2000h. Acepromazine (0.02-0.04 mg kg^{1} IV - ACP Novartis, UK) was give at 0800h, 1600h and 0000h. Pedal bone support (Styrofoam Solar Support System™/Lilypads™) was provided at the clinician's discretion. Subjects participated in the study for a maximum of 5 days, or

until euthanasia on ethical grounds was deemed necessary according to current clinical protocol.

5.2.4 Assessment Protocol

The assessment protocol used for laminitic and control horses differed from that used in chapters three and four as it was not possible to gain baseline data. Therefore, horses were monitored over a five day period or until euthanasia. Assessment included undisturbed spontaneous behaviour (with continuous and detailed observation) and examination of evoked behavioural responses. Recording commenced on the day of admittance to the hospital. One-hour behavioural samples were taken from video tapes at 8-hourly intervals (0600h-0700h; 1400h-1500h; 2200h-2300h). Direct observations were performed between 0700h and 0830h each morning (hereafter am) and between 1500h and 1600h (hereafter pm). Evoked behaviour tests were performed following am direct observation. As described in section 2.3.3.3, time of sampling reflected predicted points of minimum and maximum analgesic effect, with 0600h representing minimum action and 1400h and 2200h sample points representing possible peaks in PBZ concentration.

5.2.5 Statistical Analyses

Detailed explanations of statistical tests used can be found in section 2.5. As previously, three techniques were used to examine spontaneous and evoked behavioural results. Tests differed slightly from those used in chapters three and four due to differences in assessment protocol. The study of spontaneous disease precluded the acquisition of baseline data from each individual horse and so comparisons were made between laminitic and clinically normal horses stabled directly opposite each other, in order to account for extraneous environmental events. Three forms of statistical analysis were performed;

1. Using generalised mixed effects modelling, general analysis examined overall trends in the data to determine the effects of both 'time' and 'time of day', factors which could be influential when considering inclusion of behavioural variables in an assessment protocol.
2. Time point analysis examined the effects of administered analgesics by comparing behaviour at predicted times of minimal and maximal action. Time

point analysis was based on datasets created from a specific time point. Mixed effects models were used to determine the effect of experimental group, time (duration in the experiment) and day on these data.

3. The univariate, general and time point analyses examined behaviours separately, multivariate techniques were used to examine behaviours in combinations. Classification tree-based models examined the relative importance of behaviours in the discrimination between laminitic and control horses. In addition, discriminant analysis was used to determine the combination of behaviours providing the best discrimination between laminitic and control horses i.e. the best means for assessment of the presence and severity of laminitic pain.

5.3 RESULTS

Initial screening of the data aimed to highlight potentially significant behaviours and eliminate behaviours where problems, such as lack of data, occurred. Examples of these preliminary graphs are shown in figure 5.0.

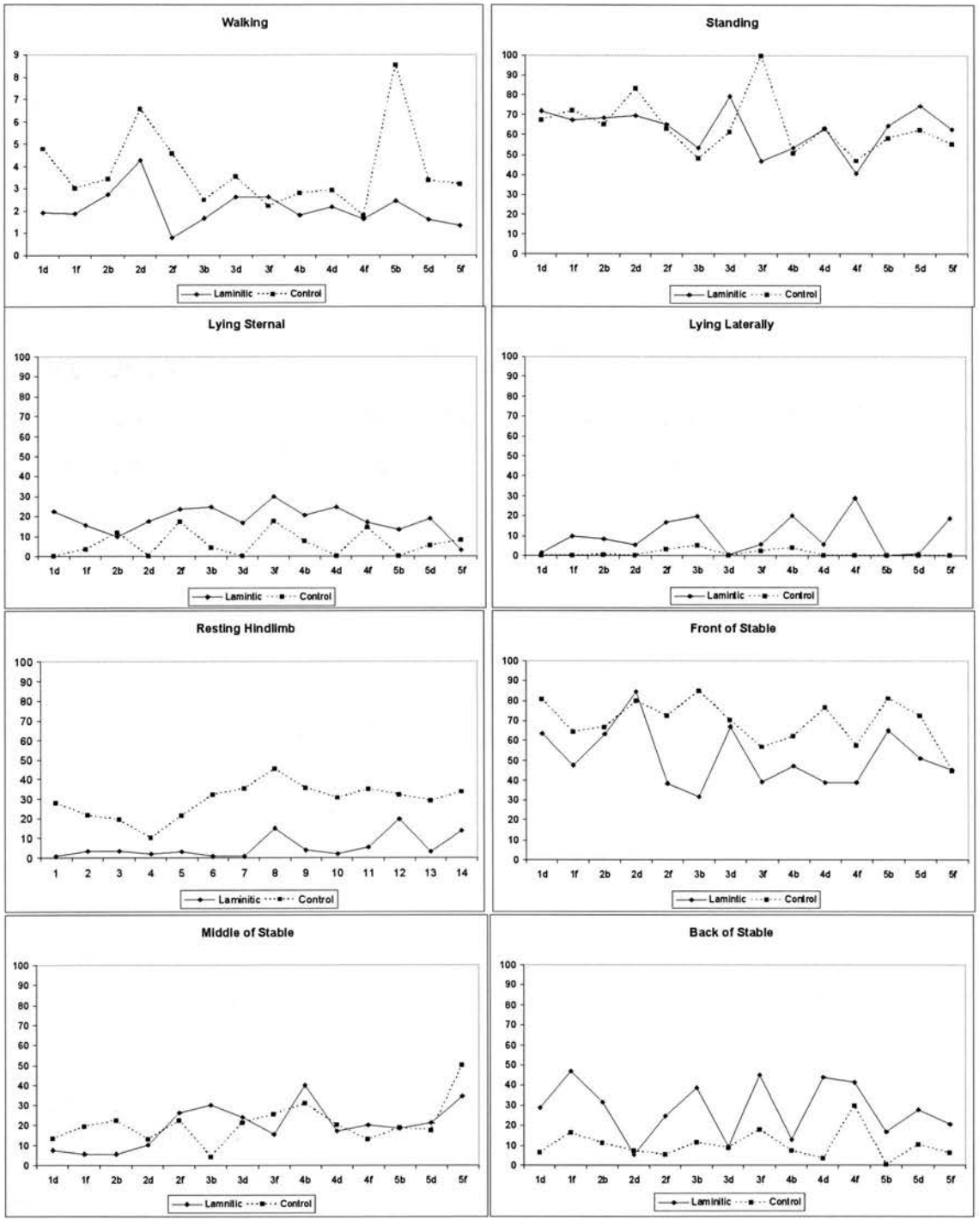


Figure 5.0 Preliminary graphical analysis of individual behavioural variables showing proportion of time spent performing each behaviour by laminitic and control horses.

Examination of preliminary graphs highlighted a number of potentially important differences in the behaviour of laminitic compared to control horses. These differences were plotted on a two-way axis to illustrate the size and direction of behavioural change. Figures 5.1 and 5.2 clearly show the differences in both state and event behaviour and table 5.2 confirms these observations with descriptive statistics.

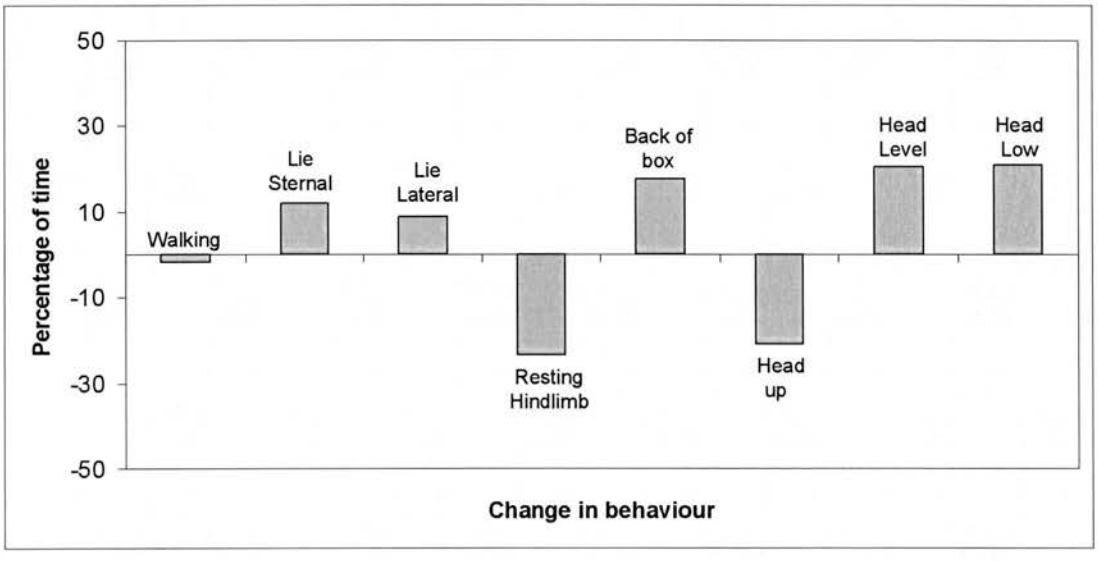


Figure 5.1 A histogram showing the exploration of differences in state behaviour in association with laminitis. The bars represent the difference in percentage of time spent performing individual behaviours between laminitic and clinically normal control horses. Positive values, such as 'back of box' indicate an increase, where as negative values indicate a decrease in proportion of time performing behaviour in association with laminitis.

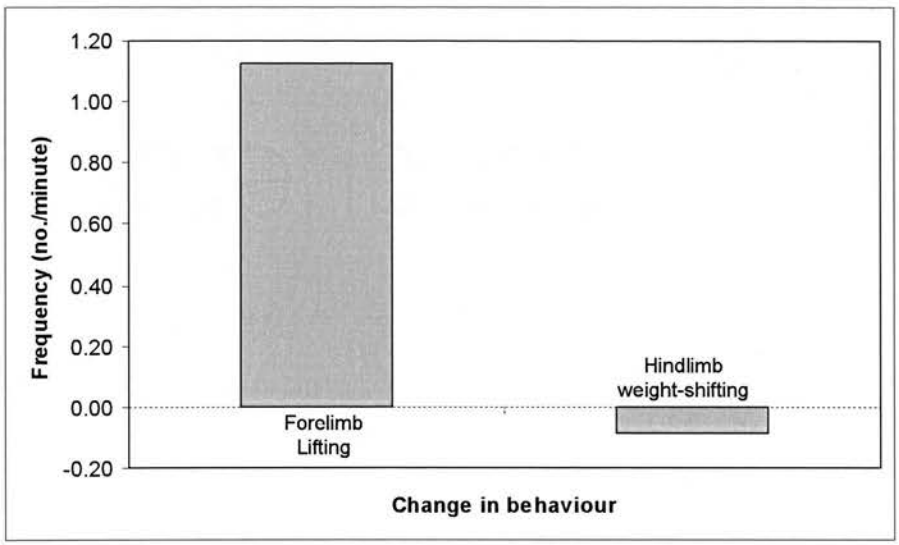


Figure 5.2 A histogram showing the exploration of changes in event behaviour in association with laminitis (see figure 5.1).

Behaviour	Laminitic	Control
Walking	0.02 ± 0.03	0.52 ± 0.57
Lie lateral	0.1 ± 0.19	0.01 ± 0.05
Resting hindlimb	0.0 ± 0.11	0.28 ± 0.23
Head up	0.45 ± 0.25	0.65 ± 0.02
Head level	0.46 ± 0.25	0.26 ± 23
Back of box	0.28 ± 0.37	0.1 ± 0.2

Table 5.2 Mean (± standard deviation) proportion of time spent performing individual behaviours by laminitic and control horses. The table shows behaviours previously highlighted as potential indicators of laminitic pain.

Exploratory analysis has shown a number of interesting differences between the behaviour of laminitic horses and that of control horses. Further analysis, using the statistical methodologies described in section 5.2.5 aimed to confirm these observations.

Significant results for univariate analysis of spontaneous behaviour are summarised in tables 5.3 and 5.4. Detailed results, including all non-significant changes and degrees of freedom, test statistics etc can be found in Appendix 4.2-4.5.

Behaviour	Group	Time	Time of Day	Day	Group*Time	Group*Day
Inattentive (rest hind)	P=0.472	P=0.429	**P=0.046**	P=0.075	P=0.940	P=0.999
Inattentive (stand)	P=0.164	**P=0.003**	**P=0.033**	P=0.071	P=0.317	P=0.999
Total lying	**P=0.024**	P=0.344	**P=0.014**	P=0.08	P=0.966	P=0.761
Head low	**P=0.046**	P=0.738	**P=0.002**	P=0.798	P=0.471	P=0.810
Stand	P=0.804	**P=0.020**	**P=0.008**	**P=0.005**	P=0.984	P=0.839
Rest hindlimb	**P<0.001**	**P=0.014**	P=0.064	**P=0.008**	P=0.093	P=0.186
Walk	**P=0.030**	P=0.621	P=0.089	**P=0.019**	P=0.926	P=0.405
Lie laterally	**P=0.006**	P=0.313	**P=0.001**	P=0.08	P=0.527	P=0.803
Grooming	P=0.467	P=0.129	P=0.693	P=0.288	**P=0.003**	P=0.203
Head up	**P=0.046**	P=0.739	**P=0.002**	P=0.798	P=0.471	P=0.810
Head Level	**P=0.049**	P=0.683	**P<0.001**	P=0.554	P=0.268	P=0.205
Front	P=0.064	P=0.068	**P=0.011**	P=0.293	P=0.419	P=0.948
Middle	P=0.897	**P=0.004**	P=0.422	P=0.283	P=0.090	P=0.271
Back	**P=0.011**	P=0.744	P=0.074	P=0.263	P=0.996	P=0.975
Weight shift	**P=0.003**	P=0.056	P=0.410	**P<0.001**	P=0.552	P=0.194
Lift Forelimb	**P=0.006**	P=0.259	P=0.692	P=0.054	P=0.061	P=0.164
Head Shake	P=0.686	P=0.732	P=0.954	**P=0.037**	P=0.706	P=0.397

Table 5.3 Significant results of general analysis of spontaneous behaviour (via CCTV).

Behaviour	Group	Time	AmPm	Day	Group*Time	Group*AmPm	Group*Day
Attentive	P=0.369	**P=0.021**	P=0.595	**P=0.019**	P=0.198	P=0.995	P=0.267
Ears forward	P=0.077	P=0.133	**P=0.01**	P=0.58	P=0.893	P=0.191	P=0.663
Ears back	P=0.796	**P<0.001**	**P<0.001**	**P=0.001**	P=0.416	P=0.852	P=0.759
Lick & chew	P=0.745	P=0.600	P=0.262	**P=0.046**	P=0.292	P=0.317	P=0.118
Tail flick*	P=0.724	P=0.750	**P<0.001**	P=0.647	P=0.117	P=0.743	P=0.385
Skin twitch*	P=0.493	P=0.285	**P=0.004**	P=0.055	P=0.695	**P=0.006**	P=0.128
Tail flick≠	P=0.874	P=0.461	**P<0.001**	P=0.633	P=0.761	P=0.699	P=0.541

Table 5.4 Significant results of general analysis of spontaneous behaviour (direct observation). * denotes occurrence analysis and ≠ level of occurrence analysis.

5.3.1 Behaviours Indicative of Chronic Pain

5.3.1.1. Spontaneous behaviour

Spontaneous behaviour monitored via CCTV camera included 10 behaviours altered in association with pain. Other, more subtle, behaviours (see Appendix 1.1) monitored through direct observations were not significantly different in laminitic compared to control horses ($P<0.076$). Whilst a number of behaviours varied significantly ($P<0.01$) between morning and afternoon sampling (ears forward/back; tail flicking; skin twitching) the lack of significant interactions between experimental group and am/pm suggested that changes are similar in laminitic and control horses. Some behaviours also varied ($P<0.047$) with time (attentive state; ears back; forelimb lifting) and day number (attentive state, ears back, lick and chew). Again, no significant interaction between experimental group and these variables suggests that changes occurring were present in both laminitic and control horses ($P>0.118$). The following sections detail the undisturbed behaviours highlighted as potential indicators of chronic laminitic pain.

5.3.1.1.1 Forelimb Lifting

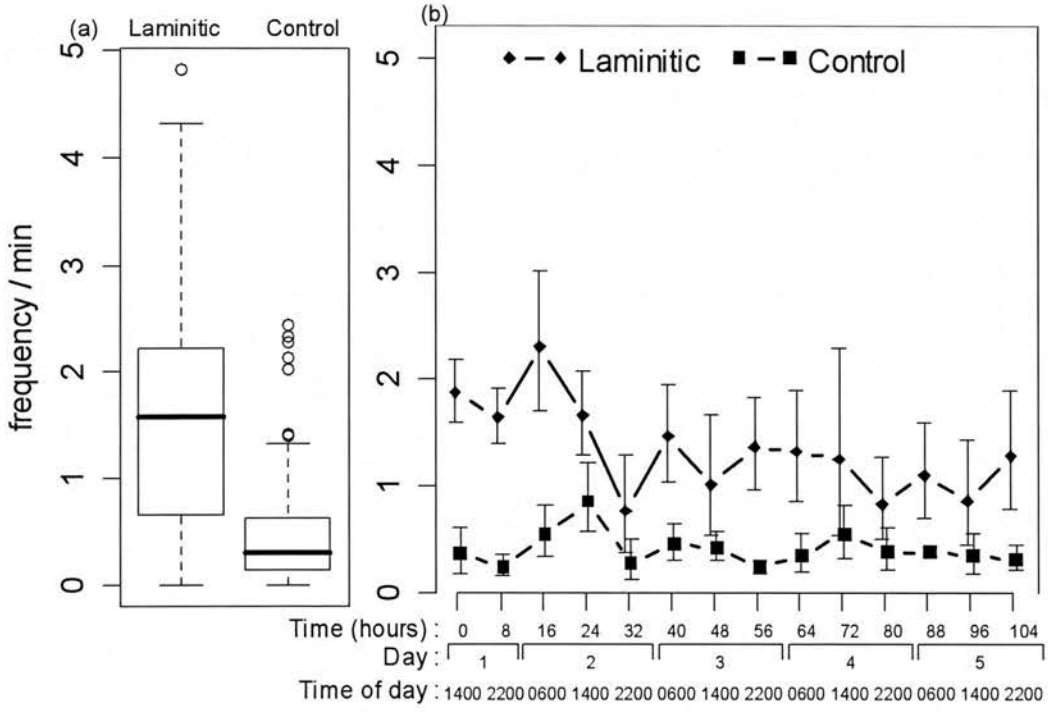


Figure 5.4 (a) Box plot of overall frequency (per minute) (±SE) of forelimb lifting in laminitic and control horses. (b) Line plot showing mean frequency (per minute) (±SE) of forelimb lifting) in laminitic (diamond, solid line) and control (square, dotted line) horses at 0600h, 1400h and 2200h over a 5 day experimental period. Descriptive statistics for line plot data were calculated from square root transformed data and then back transformed. In order to maintain clarity laminitic and control data points are offset, in time, at each point for clarity.

Square root transformed frequency of forelimb lifting was significantly greater in laminitic compared to control horses (P=0.006) as can be seen in figure 5.4(a), where there was no effect of time, day, time of day or interaction between group and time or day (P>0.054).

Time point analysis found forelimb lifting was significantly greater following intervention at both 0600h and 2200h (P<0.006). There were no significant effects of time or interaction between group and time at either 0600h or 2200h (P>0.157).

5.3.1.1.2 Resting a hindlimb

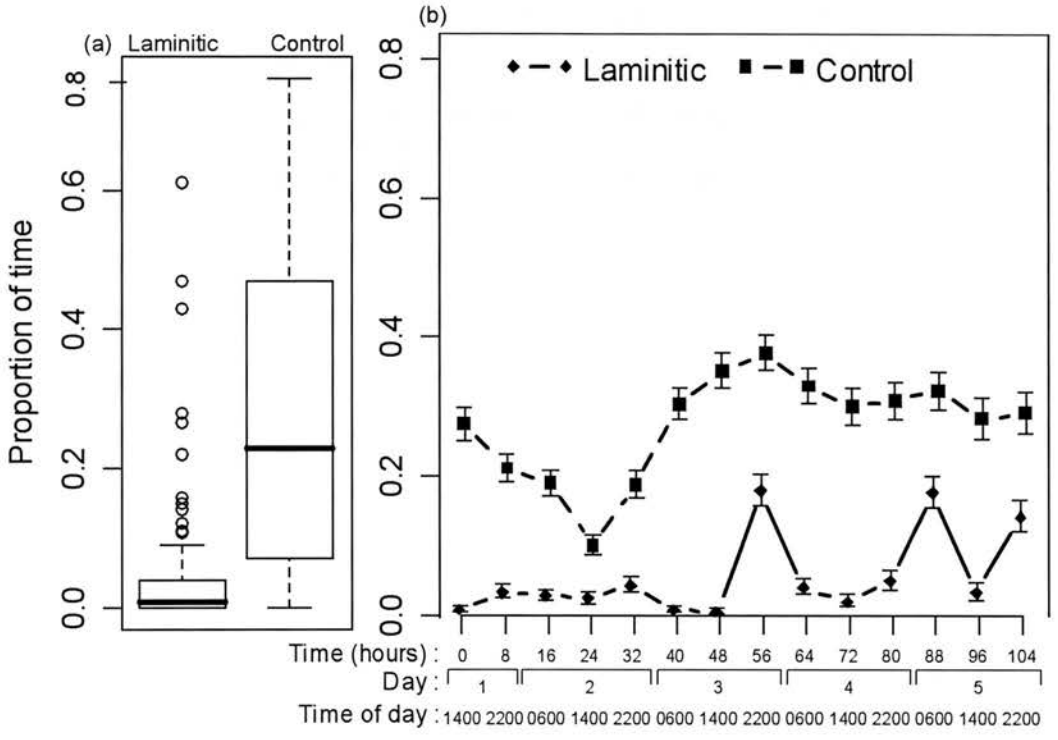


Figure 5.5 (a) Box plot of overall proportion of time (±SE) spent resting a hindlimb (adjusted for total time standing) in laminitic and control horses. (b) Line plot showing mean proportion of time (±SE) spent resting a hindlimb (adjusted for time spent standing) in laminitic (diamond, solid line) and control (square, dotted line) horses at 0600h, 1400h and 2200h over a 5 day experimental period. In order to maintain clarity laminitic and control data points are offset at each time point for clarity.

Laminitic horses spent a significantly reduced time resting a hindlimb (adjusted for time standing) compared to control horses ($P<0.001$). A significant effect of time and day ($P<0.014$) was identified but no interaction between group and time or day ($P>0.093$), suggesting that differences in behaviour at different times of day were the same in laminitic and control horses. This can be seen graphically as an increase in hindlimb resting in both groups during the study (figure 5.5b). There was no change in behaviour with time of day ($P=0.064$).

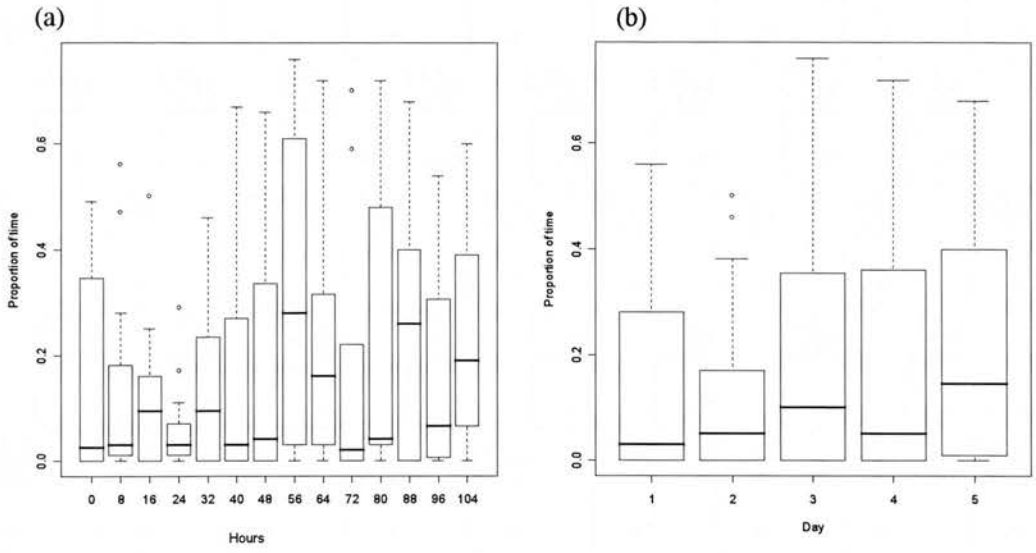


Figure 5.6 (a) Change in proportion of time (±SE) spent resting a hindlimb with time (hours in experiment) in laminitic and control horses combined. (b) Proportion of time (±SE) spent resting a hindlimb in all horses over 5 days.

Post hoc analysis, removing data from each day in turn, found that a significant day effect remained when day one, three, four and five ($P<0.006$) were excluded but not when day two ($P=0.187$). This suggests the overall significant effect of day results from differences in the data from day two. As can be seen in figure 5.6 (b) the proportion of time spent resting a hindlimb is lower on day two.

Time point analysis showed hindlimb resting was significantly reduced in laminitic horses at both 0600h and 2200h ($P<0.011$). There was no change in this behaviour over time when considering each sample point individually ($P>0.068$) but no interactions between group and time ($P> 0.114$). This behaviour was not affected by day and no significant interaction with group was found at either sample point ($P>0.08$).

5.3.1.1.3 Hindlimb Weight-shifting

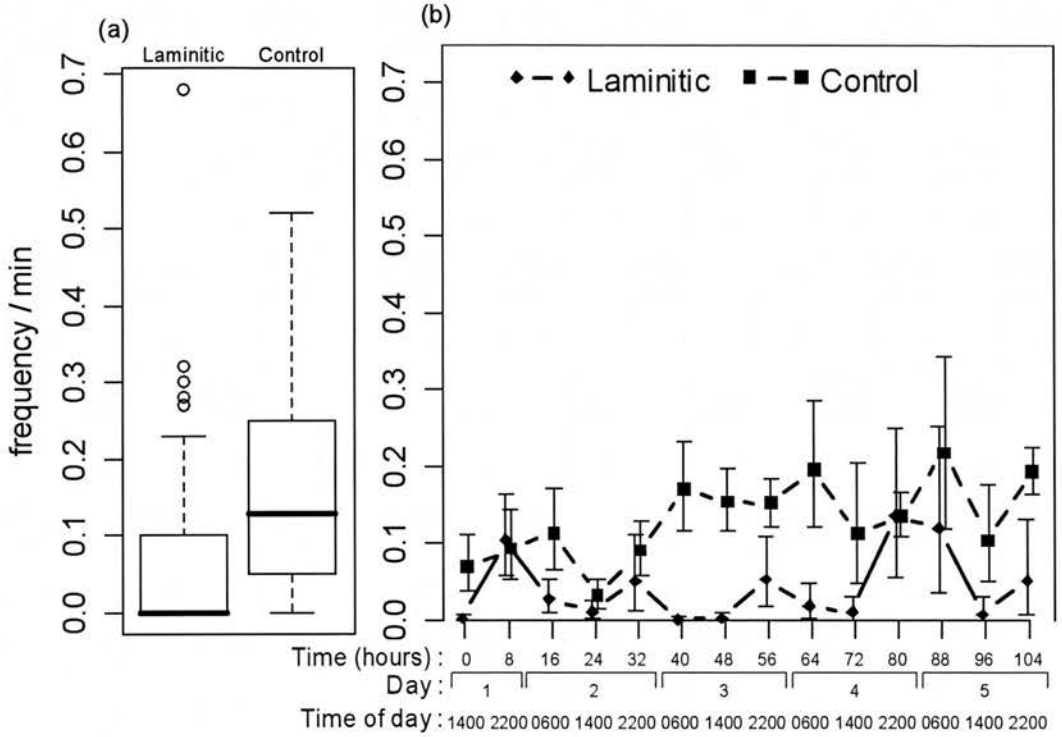


Figure 5.7 (a) Box plot of frequency (±SE) of hindlimb weight-shifting in laminitic and control horses. (b) Line plot showing mean frequency (±SE), of hindlimb weight-shifting in laminitic (diamond, solid line) and control (square, dotted line) horses over a 5 day experimental period. In line with level of occurrence analysis, '0' values were discounted and only data points where behaviour occurred were considered in the generation of this graph.

Laminitic horses showed a lower frequency of hindlimb weight-shifting than controls ($P=0.003$) as is seen most clearly in figure 5.7a. No effect of time, day, or interaction between group and time ($P<0.056$) was found. However, a significant effect of time of day was identified ($P<0.001$) and is demonstrated by the peaks and troughs seen in both laminitic and control data lines in figure 5.7b. Removal of data from time points 0600h and 2200h ($P<0.028$) caused no change in this significant affect. However, following removal of time point 1400h, no significant affect was found ($P=0.236$). This suggests, as confirmed in figure 5.8, that this effect results from a reduction in frequency of hindlimb weight-shifting at 1400h

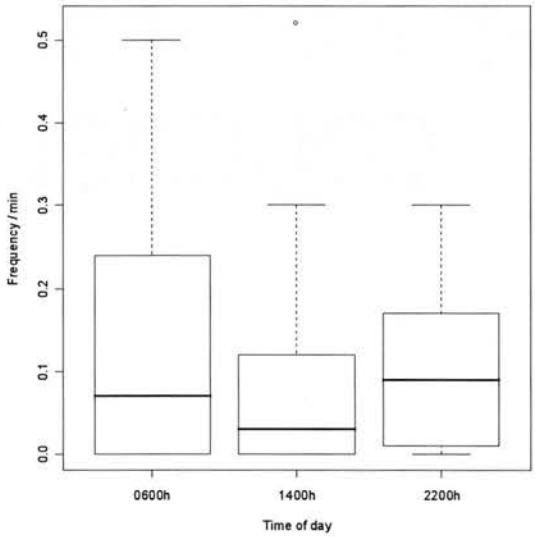


Figure 5.8 Frequency of hindlimb weight-shifting in all horses at all 0600h, 1400h and 2200h time points.

Time point analyses of hindlimb weight shifting were carried using occurrence and level of occurrence techniques as insufficient data points meant that square root transformation was not sufficient to normalise the distribution of residuals. At both 0600h and 2200h sample points, occurrence of hindlimb weight-shifting was significantly greater in laminitic compared to control horses ($P<0.048$). Level of occurrence was not significantly different at 0600h or 2200h ($P>0.285$). A significant effect of day was seen in level of occurrence analysis at 0600h ($P=0.04$). However, as no significant interaction between group and day was found ($P=0.605$), this change is occurring in both experimental groups. There was no effect of time or interaction between group and time with occurrence or level of occurrence analysis ($P>0.611$). There was no effect of time or day or interaction between group and these parameters with occurrence or level of occurrence analysis ($P>0.157$) at 2200h.

5.3.1.1.4 Walking

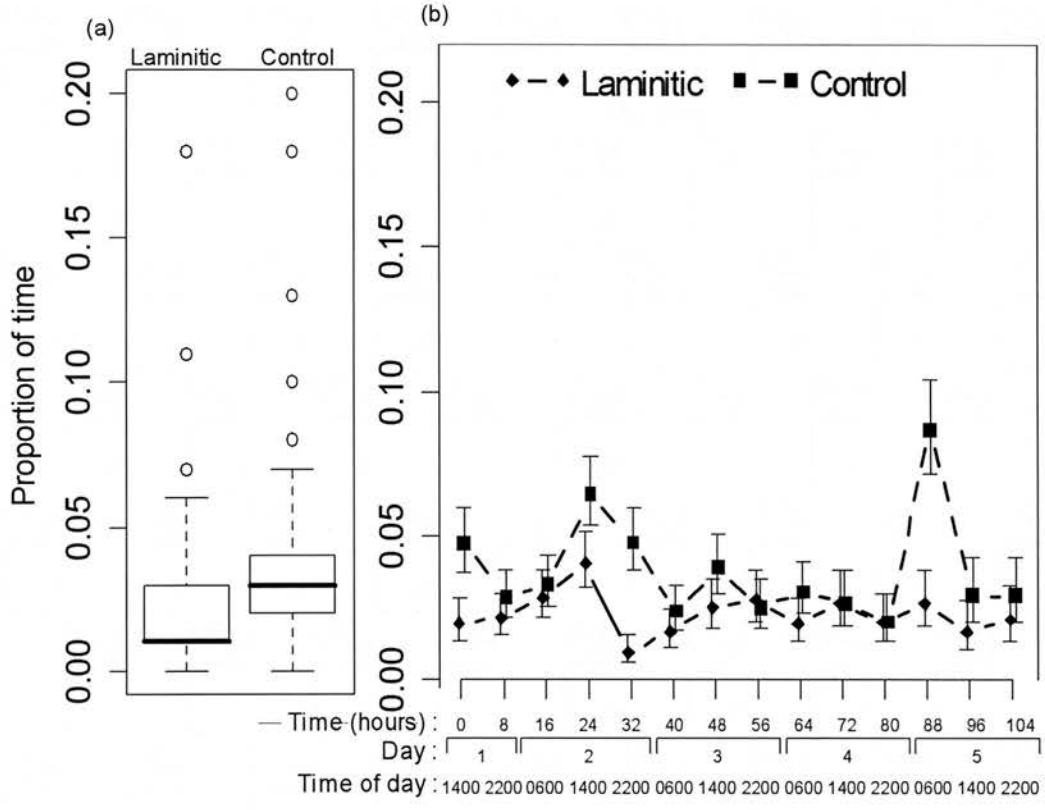


Figure 5.9 Proportion of time (±SE) spent walking in laminitic and control horses (see figure 5.3).

Proportion of time spent walking was significantly reduced in laminitic horses compared to controls (P=0.030) (figure 5.9). There was no effect of time (P=0.621) or time of day (P=0.089) and no interaction between group and time (P=0.926). However, a significant effect of day number was found (P=0.019). The lack of an interaction between group and day (P=0.405) suggested that alterations in behaviour with day were similar in both laminitic and control groups.

Post hoc analysis found that whilst excluding days one, two, three and 5 did not alter the significant day effect result, excluding data from day four returned a non-significant result for day, suggesting that day four is different from the others.

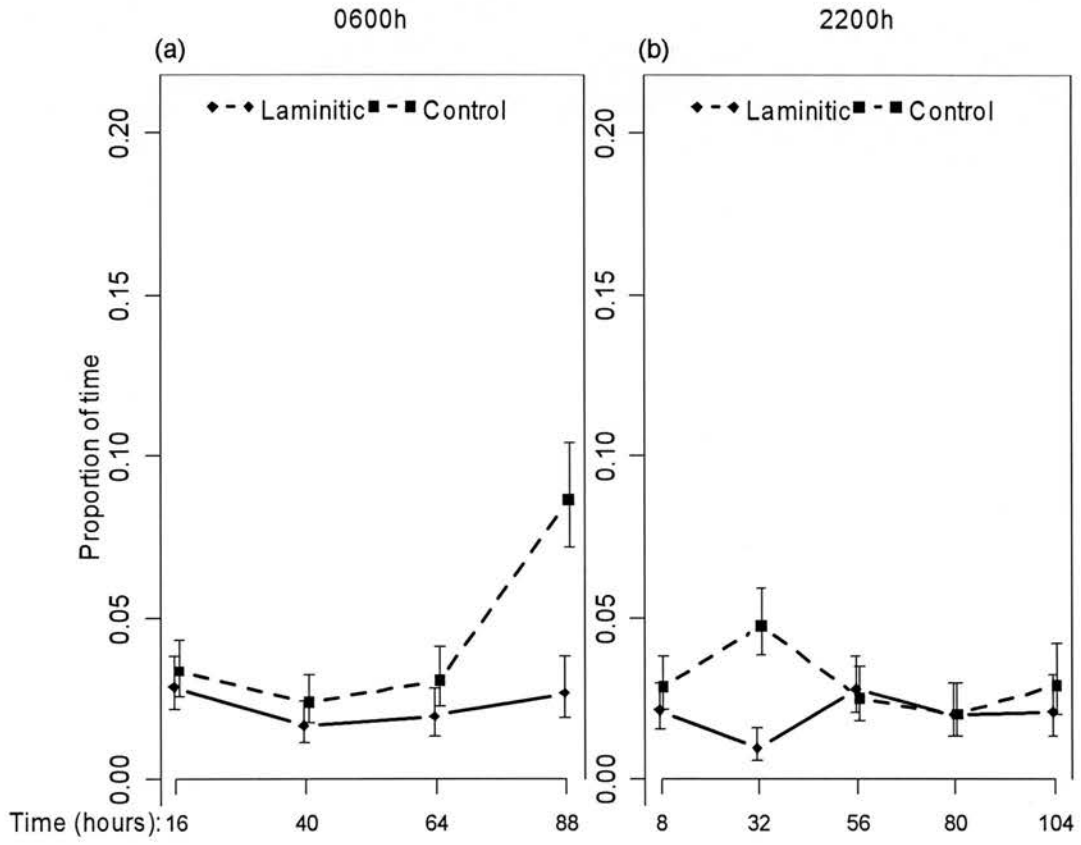


Figure 5.10 Mean proportion of time (±SE) spent walking in laminitic and control horses over five days at (a) 0600h and (b) 2200h time points.

Time point analysis (shown in figure 5.10) showed a significant difference in the proportion of time spent walking over 5 days in laminitic compared to control horses at 0600h (P=0.033), but not at 2200h (P=0.251). There was no effect of time (P=0.052; P=0.771) or interaction between group and time (P=0.211, P=0.283) at 0600h and 2200h respectively.

5.3.1.1.5 Recumbency

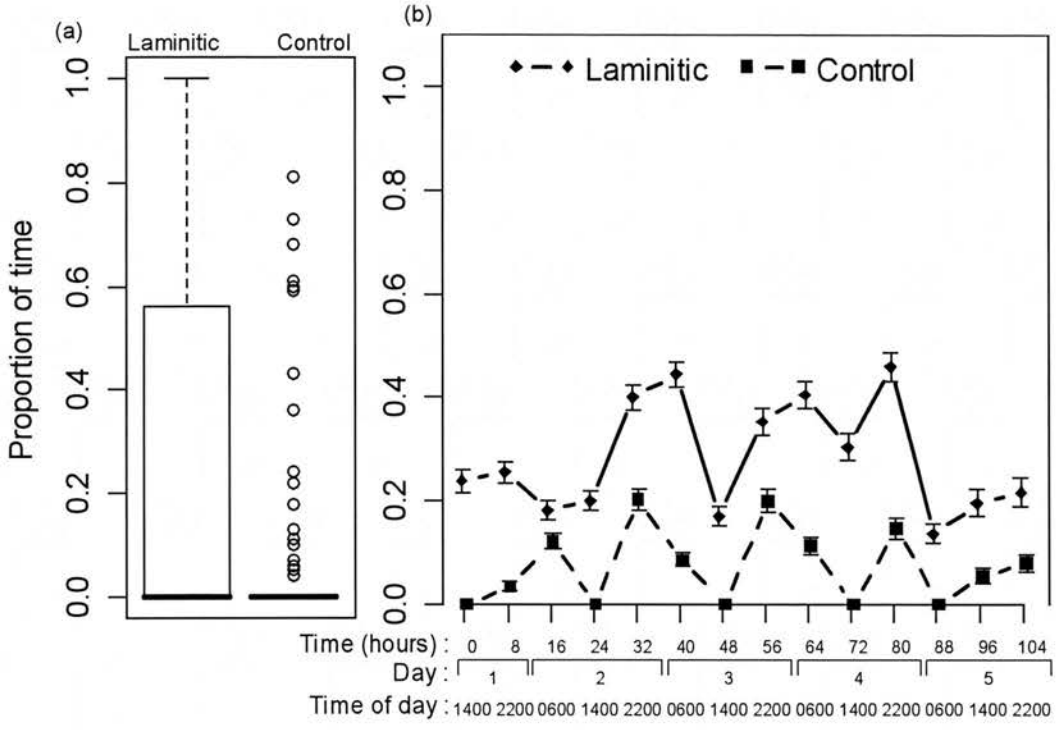


Figure 5.11 Proportion of time (±SE) spent recumbent in laminitic and control horses (see figure 5.3).

Examining the sum of sternal and lateral recumbency to determine the total time recumbent, laminitic horses spent a greater time recumbent that controls (P=0.024, figure 5.11 a and b). There was no effect of time or day and there was no interaction between group and time or group and day (P>0.08). A significant effect of time of day was found (P=0.014) and can be seen in figure 5.11b as distinct peaks and troughs in the data for laminitic and control horses. Post-hoc analysis found the removal of time points 0600h and 2200h (P<0.027) still produced a significant result, however, when time point 1400h was removed there was no significant affect of time of day (P=0.532, figure 5.12).

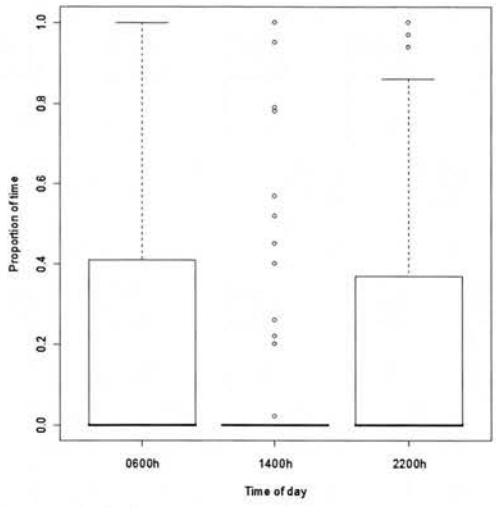


Figure 5.12 Proportion of time (±SE) spent recumbent in all horses at all 0600h, 1400h and 2200h time points.

Time point analysis found proportion of time spent recumbent was significantly greater in laminitic horses at 0600h (P=0.043) but not at 2200h (P=0.083). As can be seen in figure 5.12, control horses exhibit some level of diurnal variation in lying behaviour, with peaks generally at 2200h. This rhythm is altered in laminitic horses. There was no effect of time in study or interaction between time and group at 0600h (P>0.546) or 2200h (P>0.797).

Proportion of time spent in sternal recumbency was not significantly different between laminitic and control horses (P=0.105). However, figure 5.13 suggests that laminitic horses spend more time in sternal recumbency and lack of a significant difference may be due to the large inter-group variation in this behaviour. There was no effect of time, time of day or day and no interaction between group and time or day (P>0.148). Time point analysis also showed no significant difference between groups at either 0600h or 2200h (P>0.112). There was no effect of time or interaction between group and time at 0600h or 2200h (P>0.323).

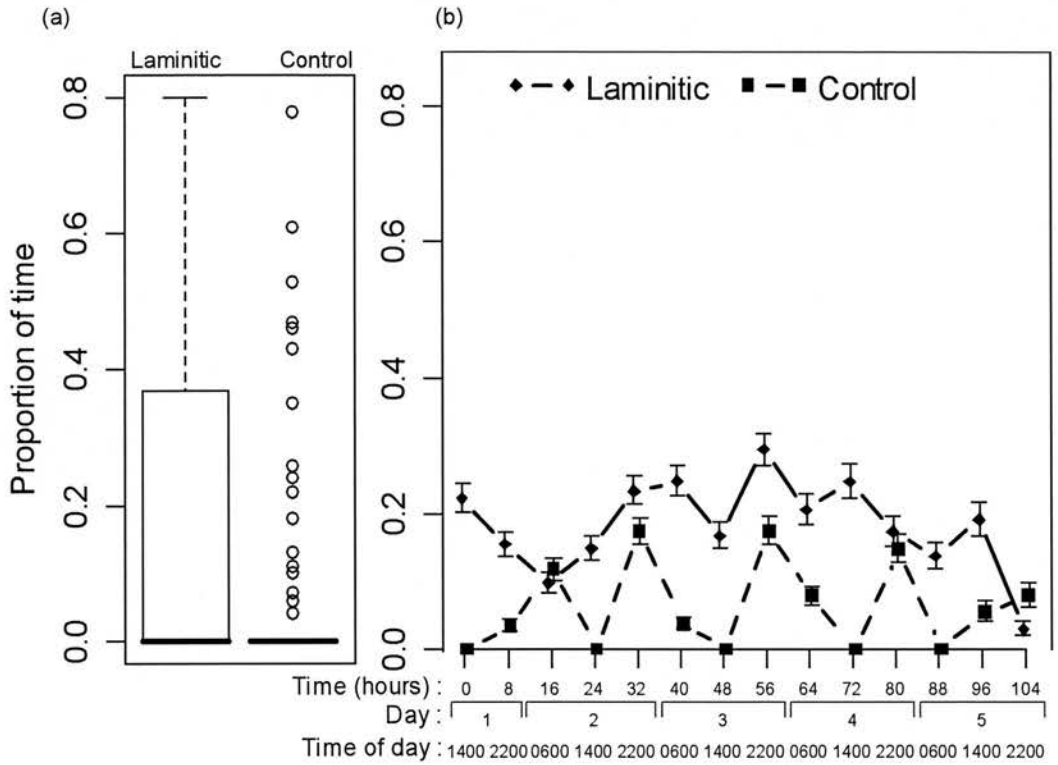


Figure 5.13 Proportion of time (±SE) spent in sternal recumbency in laminitic and control horses (see figure 5.3).

Time spent in lateral recumbency was significantly greater in laminitic compared to control horses (P=0.006). There was no effect of time, day or interaction between group and time (P>0.313). A significant effect of time of day was found (P=0.001). Time point analysis found significant differences at both 0600h and 2200h (P<0.039), with no effect of time (P>0.351) or interaction between group and time (P>0.587).

5.3.1.1.6 Head position

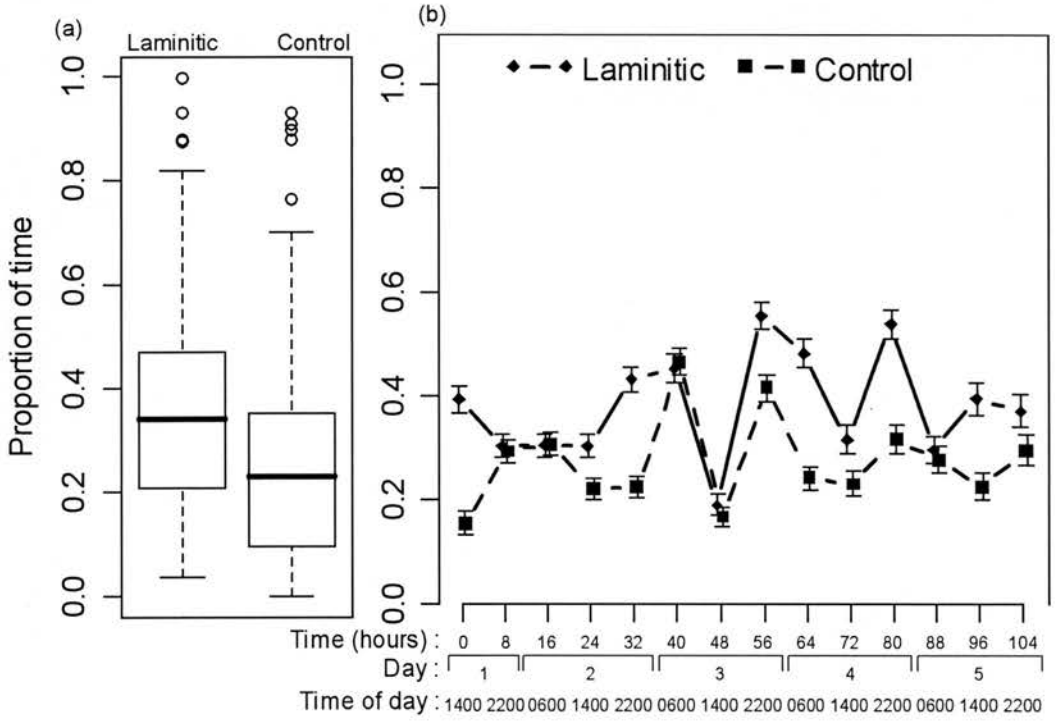


Figure 5.14 Proportion of time (±SE) spent with head low (adjusted for time spent feeding) in laminitic and control horses (see figure 5.3).

Head position behaviours were expressed as a proportion of time spent not feeding as variations in husbandry (laminitic horses were fed from hay nets, whilst control horses had hay from hay racks positioned higher up) may have effected head position. Proportion of time spent with head low (figure 5.14a) was significantly greater in laminitic compared to control horses (P=0.046). Further analysis found laminitic horses to spend greater time with their heads level with withers (P=0.049) but no difference in the time spent with head down (P=0.458). There were no effects of time, or day and no interaction between group and these variables (P>0.4). A significant affect of time of day was found (P=0.002).

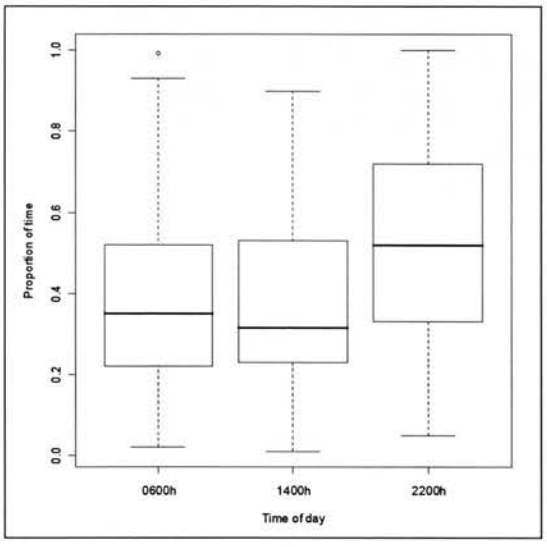


Figure 5.15 Proportion of time spent with head low in both laminitic and control horses at 0600h, 1400h and 2200h over five days.

Post hoc analysis found this significant affect remained following the removal of data from 0600h and 1400h ($P<0.02$) but was not present when 2200h data was removed ($P=0.435$, figure 5.15).

Time point analysis reinforces the *post hoc* results, finding no significant differences in head position between laminitic and control horses at 0600h ($P>0.3$) but at 2200h, time spent with head low was significantly greater in laminitic horses ($P=0.027$). This was produced by an increase in head level position ($P=0.02$) with no alteration in head down ($P=0.737$). There were no effects of time or day or interactions between group and these parameters ($P>0.058$) at either time point (0600h and 2200h).

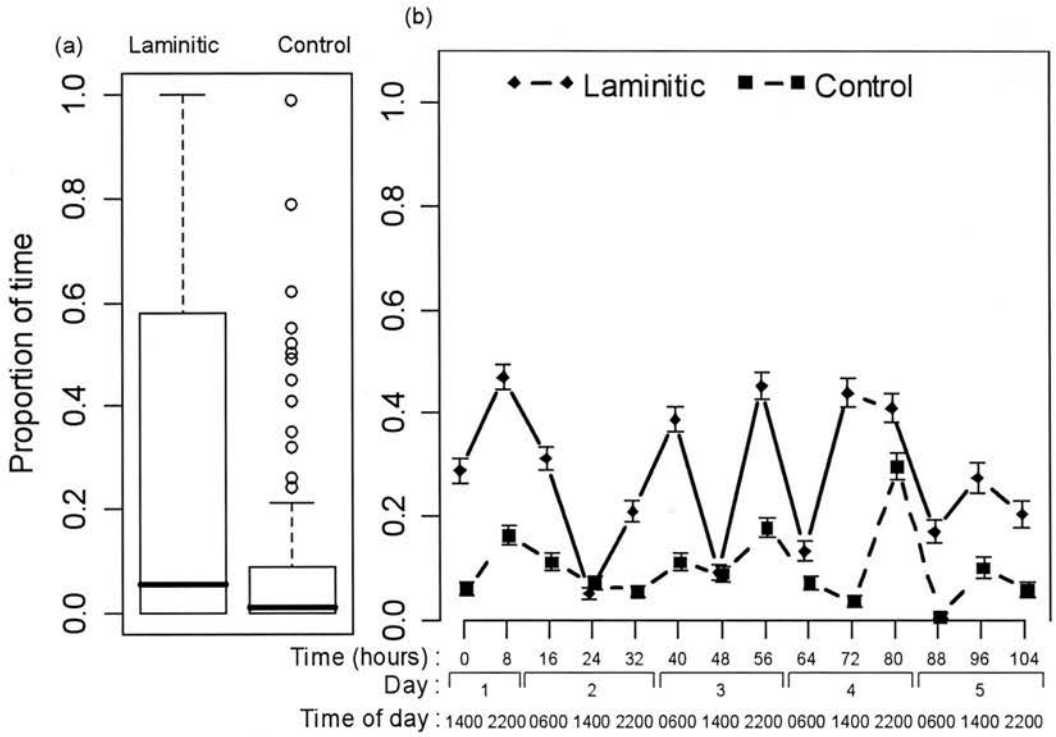


Figure 5.16 Proportion of time (±SE) spent at the back of the box in laminitic and control horses (see figure 5.3).

Laminitic horses spent significantly more time at the back of the box compared to control horses (P=0.011, figure 5.16a). There were no other significant affects or interactions with this behaviour (P>0.075). Time spent in the front or middle of the box was not significantly different between groups (P>0.065). Time spent in the front of the box was significantly affected by time of day (P=0.011, figure 5.16b) and time spent in the middle of the box was affected by time (P=0.004). Otherwise, no other significant effects or interactions were found (P>0.069).

The removal of time point 0600h still resulted in a significant time of day affect (P=0.027). Following the removal of time points 1400h and 2200h there was no significant time of day affect (P>0.113). Position in the box was not significantly different between laminitic and control groups at 0600h or 2200h. There was no effect of time or interaction between time and group at either time point (P>0.058).

5.3.1.2 Evoked behaviour

No significant difference in evoked behaviour (see Appendix 4.5) was found between laminitic and control horses (P>0.141). Evoked behaviour was consistent over the five day period (P>0.246) in all behaviours apart from level of occurrence of stepping away, which was significantly affected by day (P=0.032). Unfortunately, insufficient data points made *post hoc* analysis impossible. There were no significant interactions between experimental group and day (P>0.345) for any evoked behaviours.

5.3.2 Tree-based Analysis

As detailed in section 2.5, a number of tree-models were drawn using data from the 0600h time point on days 2-5. These time points were selected in order to incorporate undisturbed and interactive behaviour. Examination of the frequency of occurrence of behaviours within these trees aimed to gain an idea of behaviours important in splitting the two groups.

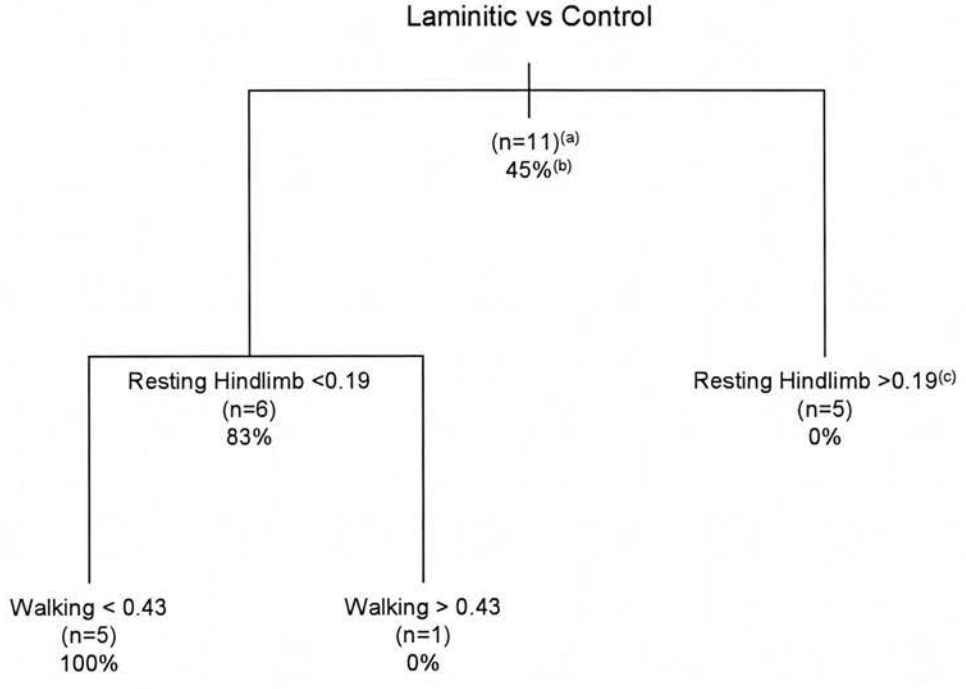


Figure 5.17 Tree-model for identification of laminitic and control horses at 0600h on day four. Where value (a) represents the total number of horses in the group and value (b) represents the percentage of laminitic animals in that group and c) represents the proportion of time spent (state behaviours) or frequency (event behaviours) of behaviour.

Figure 5.17 shows a representative tree diagram. Feeding behaviours were removed from analysis due to differences in feeding regimes. Table 5.5 shows a summary of all tree-based model results.

Behaviour	Division		
	Primary	Secondary	Tertiary
Resting hindlimb	4	0	0
Head level	1	0	0
Sternal recumbency	0	2	0
Standing	0	1	0
Walking	0	1	0
Front of box	0	1	0

Table 5.5 Behaviours identified in tree-based models as forming the primary, secondary and tertiary tree 'branches'.

The tree analysis revealed proportion of time spent resting a hindlimb to demonstrate the clearest division, forming the initial branch in 3 out of 4 trees. Locomotive behaviours, such as sternal recumbency, standing and walking all occurred at either primary of secondary branches as is shown in figure 5.17.

5.3.3 Discriminant Analysis

Discriminant analysis was performed on data from all time points on day 2. At 0600h, forelimb lifting frequency alone provided optimum discrimination between groups (89.1%). Removal of this behaviour from the analysis resulted in the summation of all leg movements providing an overall accuracy of discrimination of 78.6% (laminitic – 85.7, control - 71.4%). Removal of this parameter reduced accuracy of discrimination to 71.4%, using hindlimb resting behaviour alone (laminitic – 57.1%, control - 85.7%).

Time spent with head level showed 76.9% (laminitic – 85.7%, control - 66.7%) accuracy of discrimination at 1400h, however, if this behaviour was removed from the analyses, discrimination was no longer possible.

Combining time spent with head level and time spent in lateral recumbency correctly assigned all animals (100%) to their appropriate groups at 2200h. Following removal of these behaviours from the analysis, hindlimb resting alone gave an accuracy of discrimination of 78.6% (laminitic – 71.4%, control - 85.7%). Further removal of hind limb resting meant that discrimination was not possible i.e. it was not possible to split laminitic and control groups on the basis of the remaining behaviours.

5.4 DISCUSSION

Unsurprisingly, behaviours associated with weight-bearing, such as fore- and hindlimb movements, locomotory and recumbent behaviours were found to be altered in the presence of chronic digital pain, such as that produced by laminitis. Further to these behaviours, head position and position in the stable were also identified as important potential indicators of pain. Key behaviours are summarised in tables 5.6. and 5.7.

	Behaviour
↑	Forelimb lifting
↓	Resting hindlimb
↓	Weight shifting
↑	Recumbency
↑	Lateral recumbency
↓	Walking
↓	Head up
↑	Head level
↑	Head low
↑	Back of the box

Table 5.6 Behaviours increased (↑) or decreased (↓) in association with laminitic pain.

Tree-based Models	Discriminant Analysis		
	0600h	1400h	2200h
Resting hindlimb	Forelimb lifting	Head level	Head level +
Head level			lateral recumbency
Sternal recumbency	Leg movement		
Standing			Hindlimb resting
Walking	Hindlimb resting		
Front of box			

Table 5.7 Behaviours identified as important in the discrimination between painful (laminitic) and 'pain-free' (control) horses. Behaviours identified as important in the discrimination between painful (laminitic) and 'pain-free' (control) horses.

5.4.1 Behaviours Indicative of Chronic Hoof Pain

5.4.1.1 Forelimb lifting

Forelimb lifting has been associated with laminitic pain, initially forming part of the Obel grading system (Obel 1948), in which the behaviour was described as "paddling". In a paper, examining behavioural and physiological responses to NSAID therapy in laminitic horses, Reitmann et al. (2004) found frequency of *'weight-shifting between contralateral limbs'* to be decreased following provision of analgesia. In the current study, general analysis found increased forelimb lifting in association with laminitis,

representing an abnormal behaviour, being at low levels or absent in clinically normal horses. Additionally, the behaviour was highlighted as an important discriminator between groups in discriminant analysis. Interestingly, when individual time points are evaluated, statistical significance was only evident when forelimb lifting was measured at 0600h and 2200h. A possible explanation for this finding is that increases in PBZ analgesic effect at 1400h are reducing behavioural differences at this point. Alternatively, personal observation suggests increased activity in the hospital environment at this time may be altering behaviour. Forelimb lifting may be associated with resting behaviour and therefore may occur less in times of high environmental stimulation. Stress-induced analgesia is a recognised phenomenon, characterised by the reduction or elimination of pain behaviour in situations of fear (Sneddon et al. 2003). Additionally, research shows further suppression of behavioural responses to pain in situations of conflicting motivation, where attention is shifted towards the environment or food (Gentle & Corr 1995; Wylie & Gentle 1998; Gentle 2001). Degree of modulation is affected by motivation levels (Gentle 2001), so it might be expected that the effects would be reduced with time as animals become habituated to the environment. Change was not apparent in the results presented here, suggesting either fear is not having a significant motivational effect in this case or that the horses are not fully habituating to the environment during the course of the study.

5.4.1.2 Hindlimb resting and weight-shifting

Laminitis predominantly affects the forefeet (Hood et al. 2001), resulting in the contra-lateral shifting of weight between feet (as described above) and decreased weight-bearing on the forelimbs by repositioning and increased weight-bearing by the hind feet. This clinical observation is supported by this data. There was no effect of time of day, as seen in forelimb lifting frequency, with hindlimb resting consistently reduced at each of the time points in the laminitic group. The decrease in time spent resting a hind limb in laminitis shows a reduction in the performance of normal maintenance behaviour. Resting and 'sleep standing' behaviour in horses is generally associated with weight bearing on three legs (McDonnell 2003). The changes reported here suggest that the horse is not able to rest satisfactorily. The increase in hind limb resting behaviour over time, seen to the same extent in both laminitic and control horses, suggests possible

environmental influences on behaviour, most probably as a result of habituation to the novel hospital environment. Interestingly, whilst the importance of forelimb lifting has been highlighted by its use in the Obel grading system (Obel 1948) and as an indicator of laminitic pain in other studies (Rietmann et al. 2004), decreased hindlimb resting does not appear to have been reported, despite its clear importance in the discrimination between laminitic and normal horses in the current study.

During periods of hindlimb resting, normal horses commonly shift weight from one hindlimb to the other (Eager, personal observation). Results of the current study suggest that this behaviour is decreased in association with laminitis. Clinical signs are generally more predominant in the fore feet, commonly affecting the hind feet less severely. All of the laminitic horses in the current study predominantly showed forelimb lameness, however, whilst less common, laminitis also affects the hindlimbs. Whether the decreased hindlimb resting and weight-shifting identified here would be altered in animals suffering predominantly hindlimb pain requires further investigation.

5.4.1.3 Recumbency

Total time spent recumbent was increased in association with laminitis, predominantly characterised by a significant increase in lateral recumbency. However, graphical results suggest that laminitic horses tend to also spend more time in sternal recumbency. The lack of a significant difference in the time spent in sternal recumbency may be due to the large inter-group variation in this behaviour. These results confirm the suggestion that increased lateral recumbency is associated with chronic pain states (Matthews 1992). A clear diurnal rhythm can be seen in control horses; however, this rhythm is disrupted in laminitis, with less clear peaks and troughs. In normal horses lateral recumbency predominantly occurs between the hours of 00.00 and 04.00 (Boyd et al. 1988), spending around 140 minutes per night recumbent (Raabymagle & Ladewig 2006), approximately 29% of which is spent in lateral recumbency.

5.4.1.4 Head Position

In general, laminitic horses spent more time with their head in the low position than control horses. As in previous chapters, increased positioning of the head '*level with the*

withers', but not *'below the withers'*, was significantly different between groups. Showing significant differences in general analysis and appearing in tree-model and discriminant analysis, time spent with head level is suggested as an important, 'non-limb' related indicator of chronic pain.

5.4.1.5 Position in the Loose Box (stable)

Position in the stable as an indicator of pain has been discussed in chapters two and three. In the current chapter, laminitic horses spent a greater time towards the back of the box. This result is in line with the findings of Price et al. (2003), where a trend for increased time spent at the back of the box seen in post-surgical arthroscopy patients. Pritchett et al. (2003) incorporated a score of position in the stall into their composite postural score (including stall position, head position, ear position, locomotion and gross pain behaviour) for exploratory celiotomy patients. Standing in the middle of the stall, facing backwards was given the greatest score, representing the highest degree of pain, with standing at the door watching given the least. Following surgery, horses had altered posture scores compared to controls and horses having undergone only general anaesthesia. However, as the score allocated for this behaviour was summed with others such as head position, it was impossible to determine the extent to which each individual behaviour (i.e. stall position) was altered in the surgical group. This change in behaviour may represent a reluctance to engage in the external environment and preference to remain withdrawn from surroundings. Proportion of time spent at the back of the box showed considerable variability in laminitic, and to a lesser extent, control horses. During days 1-3 these changes appeared to be diurnal, with decreased times at the back of the box at 1400h, however during days five and six no clear diurnal rhythm was seen. These changes may again be attributable to stress-induced pain modulation due to increased environmental stimuli at 1400h and may have been reduced towards the end of the study period as the horses became more habituated to their surroundings. Identifying possible changes in behaviour or behavioural rhythms over a period of hospitalisation is of particular importance when clinicians are monitoring disease progression. Observed changes may not represent clinical improvement but 'normalisation' of parameters or alteration in response to external stimuli.

5.4.2.6 Diurnal changes in behaviour

General and time point analyses have identified a number of behaviours showing significant diurnal changes in laminitic and control horses, which may result from intrinsic behavioural rhythms, the effects of external environmental stimuli or variations in analgesic action. This is of importance to future study design as it illustrates the requirement for continuous monitoring and sampling at regular intervals, when evaluating, some, but not all behavioural adaptations. The eight-hourly sample points used in the current study are not sufficiently frequent to gain detailed information on diurnal alterations in behaviour, a thorough understanding of the effects of these rhythms and the modulation of such rhythms in association with pain is required when considering potential behaviours for inclusion in a pain assessment protocol. Additionally, the reduced differences between groups at particular times of day emphasises the need to monitor a number of behaviours concurrently.

5.4.2.7 Disease progression

The study of spontaneous disease, as is the case in the current work, means that it is impossible to control disease progression. The current study assumed a 'steady state' over the five day experimental period. Therefore, changes in disease status represent a confounding factor, with some animals showing clinical improvement and some deterioration during the study.

5.5 CONCLUSIONS

The study reported here shows consistent deviations from normal behaviour in laminitic horses. Traditionally, laminitic horses have shown a poor response to anti-inflammatory analgesics, a viewpoint which is confirmed by the results reported in this chapter. Further work improving and refining analgesic protocols and pain management techniques is vital to the improvement of welfare in the laminitic horse. Previous work has identified significant variation in the assignment of Obel and clinical grading scores (Viñuela-Fernández et al. 2007) by veterinary students and clinicians, which may result in sub-optimal management of laminitis pain. The requirement for validated, objective tools for the assessment of laminitic pain is great and would not only improve the accuracy of pain assessment and therefore efficacy of management, but also be imperative in the development of analgesic therapy. The results of the current study provide objective markers of laminitic pain for use in future scientific research and may also be integrated into clinical assessments of laminitic patients.

Further work is required to assess the relationship between the degree of behavioural adaptation and the severity of both disease and pain severity. Examination of the correlation between spontaneous behaviour, subjective scoring/grading systems, radiographic images and quantitative sensory testing may be of use in this work. Additionally, the combination of data for key behaviours in order to form an 'overall pain score' and the assessment of such a score will ease clinical identification and monitoring of laminitic pain.

CHAPTER SIX

GENERAL DISCUSSION

6.1 INTRODUCTION

The following chapter presents a discussion of the key behavioural changes identified in association with acute and chronic pain resulting from sedative and anaesthetic drugs and hospitalisation will be discussed. Furthermore, the methodology used in the studies reported in this thesis is discussed and comparisons made with other research. Requirements for the development of an 'equine pain score' are discussed, including the design of such scores in the field of equine pain and the implication of the results of the present studies. A summary of the thesis chapter results is presented in table 6.1, showing the qualitative behavioural changes found to be associated with both acute post-surgical and chronic laminitic pain states in the horse.

Behaviour type	Behaviour	Acute post-surgical pain		Chronic digital pain
		SS	GA	Laminitis
Spontaneous	Head up	↓	-	↓
	Head down	-	-	-
	Head level	↑	↑	↑
	Head low	↑	-	↓
	Head shaking	-	↑	-
	Standing	-	-	↓
	Walking	-	↑	↓
	Resting hindlimb	-	-	↓
	Recumbency	-	-	↑
	Lateral recumbency	-	↑	↑
	Sternal recumbency	-	-	↑
	Forelimb lifting	-	↑	↑
	Hindlimb lifting	-	↑	-
	Hindlimb weight-shifting	↑	↑	↓
	Limb movement	-	-	↑
	Back of box	-	↑	↑
	Front of box	-	↓	↓
Evoked	Ears back	↑	↑	-
	Step away	↑	↑	-
	Head down turned towards handler	↑	-	-

Table 6.1 Summary of spontaneous and evoked behaviours identified as indicative of acute post-surgical (standing surgical anaesthesia castration (SS) and general anaesthesia castration (GA) and chronic digital (laminitis) pain. This table includes behaviours significantly different between treatment groups in univariate general analysis and highlighted as important in multivariate tree-based models and discriminant analysis.

6.2 EQUINE PAIN BEHAVIOUR

6.2.1 Pain state specific and general pain behaviour

The work reported here examined pain behaviour in both acute post-surgical and chronic laminitic pain state. In table 6.1, both pain type specific (occurring only in the horses suffering acute pain) and general (for example only occurring in both acute and chronic pain states) behavioural changes can be seen. Differences in the responses of horses in acute and chronic pain are considered to reflect differences in the nature, duration and severity of pain experienced in each model, suggesting different motivational or coping strategies for these types of pain.

The identification of pain 'type-specific' behaviours in the present results suggests that the examination of evoked behavioural responses to a standardised interaction test is particularly useful for recognition and assessment of acute post-surgical pain. Conversely, in the assessment of chronic laminitic pain, the monitoring of undisturbed spontaneous behaviour is more effective highlighting the importance of pain 'type-specific' indicators.

The responses of animals to acute pain are generally include escape and avoidance behaviours (Sanford et al. 1986) and in some cases, defensive behaviour (Zimmerman 1986). Acute post-surgical pain, in the present studies, resulted in short-term, protective behavioural responses, to a normally non-threatening situation but had little effect on the animal's undisturbed behaviour. This defensive reaction may stop the observer or approaching human from possibly inflicting more pain. Having undergone a stressful surgical procedure, castration patients may be expected to show increased nervousness in the presence of an observer and the results may reflect an element of anticipation of further negative experience. However, control horses received identical IV injections and manipulations (other than castration itself) and did not show these behavioural changes. If there was a significant anticipatory component, changes would be expected in the control animals as well.

Chronic laminitis pain was more accurately characterised by long-term behavioural changes, which, it is suggested, appeared to improve the horse's ability to cope with

persistent pain. Postural changes are apparent in a number of animal models of both chronic and acute pain, for example one-legged standing in association with sodium urate induced arthritis in the chicken (Gentle & Corr 1995). The decreased movement, increased resting and reduction in weight-bearing on the affected limbs may represent the animal's attempts to minimise pain and assist healing (Hansen 1997). Behaviours associated with chronic pain are often taken as the result of associated depression or 'learned helplessness' (Zimmerman 1986), with animals becoming apathetic or unresponsive. This may explain the lack of a significant evoked behavioural responses to interactive testing in the laminitic animals.

The differences in behavioural strategy in response to acute and chronic pain may also reflect the severity of pain experienced. Rutherford (2002) suggests evoked behavioural tests to be of use for the identification of mildly painful situations, inducing a more noticeable response where spontaneous behaviour remains unchanged. It has been suggested that castration is not a painful procedure in the horse and does not warrant provision of analgesia (Green 2001). These comments instigated debate in the veterinary press (Capner 2001; Johnson 2001; Harris 2001; Jones 2001; Flecknell et al. 2001) and confirm the findings of Price et al. (2002) who suggest a general lack of consensus within the veterinary profession as to the pain severity attributable to specific procedures. The changes seen here in evoked behavioural responses following castration indicate a level of pain or discomfort is associated with this procedure. The limited changes in spontaneous behaviour identified here are consistent with either difficulties in the detection of pain in association with castration or the absence of significant pain. Horses undergoing castration in these studies were provided with pre- and post-operative analgesia, which may have effectively relieved some (therefore limited changes in spontaneous behaviour) but not all (highlighted by changes in evoked behaviour) post-surgical pain. Examination of the behavioural responses to a range of pain severities, as is described by Molony et al. (2002), may help determine the importance of evoked behavioural responses for the assessment of acute castration pain and of spontaneous behaviour for the assessment of chronic laminitis-associated pain is related to pain severity or nature of pain.

Laminitis is a systemic disease and therefore horses are likely to not only experience pain but also a level of sickness, which could influence their behaviour in a manner undetermined by this research.

In addition to potentially pain 'type-specific' specific behaviours, changes in behaviour general to both control and treated horses were seen. For example, the proportion of time spent with head level to withers was increased in association with both acute post-surgical and chronic pain. Position in the box may also be a general indicator of pain, with increased time spent at the back of the box being particularly important in relation to laminitic pain and the pain following castration under general anaesthesia. Increased time at the back of the box was not associated with pain after surgical castration in the standing position, however, increased time in the front of the box was associated with sedation and therefore may have masked or over-ridden potential pain related changes.

6.2.2 Behavioural effects of anaesthetic and analgesic protocols

Both standing surgical anaesthesia (induced through the intravenous injection of detomidine and butorphanol in combination) and general anaesthesia (induced through IV ketamine and maintained on inhalational halothane), significantly affected behaviour, as is summarised in table 6.2.

Standing surgical anaesthesia		General Anaesthesia	
↑	Inattentive	↑	Head low
↑	Grooming	↑	Inattentive behaviour
↑	Stamping	↑	Hindlimb resting
↑	Hindlimb lifting	↑	Exploratory behaviour
↑	Front of the box	↑	Ears sideways
↑	Head down	↑	Leg movements
↓	Weight shifting	↓	Recumbency
		↓	Head up
		↓	Ears forward
		↑	Head up - towards handler (evoked)
		↑	Exploring handler (evoked)
		↑	Head down – straight (evoked)
		↑	Stamping

Table 6.2 Changes in behaviour in association with standing surgical and general anaesthesia. ↑ indicates an increase in behaviour in association with pain. ↓ indicates a decrease in behaviour in association with pain.

These changes have been thoroughly discussed in their corresponding chapters however; they have implications for pain assessment which will be mentioned here. From these results it is clear that anaesthetic and analgesic drugs significantly alter equine behaviour in a number of ways. Studies which do not control for such behavioural effects (Raekallio et al. 1997a; Raekallio et al. 1997b; Price et al. 2003) may therefore be confounded, as discussed in sections 3.4.5 and 4.4.3. A greater awareness of the quantitative behavioural effects of various anaesthetic and analgesic agents is vital for the accuracy of pain assessment in animals. However, whilst it is not practical to study all possible combinations of agents, care must be taken when generalising the behavioural effects and it should also be remembered that the affects of anaesthetic and analgesic drugs may be different in pain-free compared with horses in pain.

6.3 CLINICAL PAIN ASSESSMENT: FORMULATION OF A 'PAIN SCORE'

Behaviour has been used as an indicator of clinical equine pain in a number of studies (Johnson et al. 1993; Raekallio et al. 1997a; Raekallio et al. 1997b; Price et al. 2003; Pritchett et al. 2003; Rietmann et al. 2004) and reviewed by Ashley et al. (2005) . The methodology for the recording of this behaviour has however, varied significantly resulting in severe difficulties in the comparison of results. The present studies aimed to objectively identify pain-associated behaviour through the examination of a wide range of behaviours, removing observer bias and subjective assumptions as to the importance of a particular behaviour. Similar techniques, examining duration and frequency of behaviour have been used for the identification of pain behaviour in other species (Molony et al. 1993; Molony et al. 1995; Graham et al. 1997). However, in most of the recorded equine clinical pain literature a limited range of behaviours has been recorded, often subjectively and as a component of a 'pain score'. The results of the current study highlight some of the problems in the development of such scoring systems, including the effects of subjective assumption of what constitutes pain behaviour, either through the grouping of 'pain behaviours' or by providing weighting for a set of behaviours in a scoring system.

6.3.1 What is 'pain behaviour'? Subjective assumptions and objective analysis

Limitations of subjective univariate scoring systems such as the SDS or VAS have led to the development of composite scores (Firth and Haldane, 1999). Composite, multivariate scores, have been implemented in human medicine and are associated with improved accuracy and more consistent correlation with subjective assessment of the clinical pain experienced. A number of such composite scoring systems have been developed for use in veterinary medicine (Raekallio et al. 1997b; Firth & Haldane 1999; Pritchett et al. 2003), however, these systems are generally used without testing of repeatability or validation.

Raekallio et al. (1997a; 1997b) and Pritchett et al. (2003) used composite scores based on an NRS (see section 1.5.1.1.) to assess pain following arthroscopy and exploratory celiotomy respectively. No evidence was provided as a basis for these scoring systems. In these studies, 'head above withers' was associated with no pain, head level with moderate pain (a score of 2 or 3) and 'head down' with severe pain (score 3 or 4). The objective research reported here, performed without subjective assumptions, found 'head level' to be a general indicator of pain, where as 'head down' was associated with sedation or anaesthesia, which is in disagreement with these scoring systems.

This problem was also highlighted by Hardie et al. (1997), having examined the use of a composite behavioural score and objective behavioural analysis for the assessment of post-operative pain following ovariohysterectomy in bitches. Conclusions were found to vary significantly dependant on the method of assessment. For instance, the composite NRS found no difference in pain levels between bitches given post-operative oxymorphone and those given placebo, whereas objective behavioural analysis found behaviour of the oxymorphone group returned to control values more quickly than placebo animals. The authors offer a number of possible explanations including increased inter-observer variation when using the score, lack of sensitivity when assessing certain behaviours, the inclusion (and high weighting) of behaviours seen commonly in healthy animals and the lack of behaviours which were considered important in objective analysis.

The results of both the current studies and that of Hardie et al. (1997) suggest that objective behavioural analysis provides a more accurate and thorough view of changes in pain-associated behaviour, than many composite scoring systems. Interestingly, when using VAS scores based on previously identified behavioural changes to study castration pain in lambs, Thornton and Waterman-Pearson (1999) found similar results to more objective studies (Mellor & Murray 1989a; Molony et al. 1997) suggesting it may not always be necessary to perform long and cumbersome behavioural observations (Rutherford 2002), as long as subjective scores are based on previously validated criteria.

6.3.2 Importance or 'weighting' of behaviours in relation to pain severity

The manner in which behaviours are allocated a 'score' during the development of pain assessment protocols also warrants some discussion. It may be imagined that within a group of behavioural variables identified as potential indicators of pain, some behaviours will have a greater relative importance than others. The scoring systems developed by Raekallio et al. (1997a; 1997b) are good examples of the subjective assignment of weightings to particular behaviours. In this work 'head down' was given a higher pain score than 'head level', which, according to the results of the current study, may not reflect behavioural change in association with pain severity. In the current studies, the use of tree-based models aimed to identify the relative importance of each behaviour in the discrimination between animals 'in pain' and 'pain-free'. Techniques such as this may give an indication of potential weightings within a composite pain scale. For example, the results of the tree-based model analysis for the comparison of laminitic and control horses found resting hindlimb to be the most efficient variable for dividing the two groups on four out of five days i.e. it splits the groups most accurately. It could then be suggested that reduced time spent resting a hindlimb is an important indicator of laminitic pain and therefore should score more highly than increased frequency of forelimb lifting for instance. However, validation of behavioural responses through the objective examination of changes in these behaviours in association with varying pain severities (Molony et al. 2002) might provide an approach for future studies.

6.3.4 Grouping of individual behaviours to form a composite score

Pritchett et al. (2003) used objective recording of state behaviour (in terms of duration) from video recordings to compare behaviour of post-surgical exploratory celiotomy patients, anaesthetised animals and control animals. However, following objective recording, behaviour was subjectively assigned to one of 4 groups as shown in table 6.3.

Category	Behaviour	Description
Active	Eat	Eating hay placed in the stall
	Defecate	Lifting the tail and evacuating faeces
	Drink	Drinking water from the bucket
	Flick	Rapid, repetitive flicking of the tail while standing
	Lick	Licking the salt block
	Nose	Investigating the walls, floor or water bucket wile taking strides or standing.
	Paw	A repetitive action with the forelimb extended forward and then drawn back with the ventral toe dragging
	Roll	Lying down and rolling
	Scratch	Rubbing any part of the body against the side of the stall or the front leg or raising the hind leg to scratch the head
	Shake	A shake that involves the whole body
	Toss	Tossing, shaking or stretching the head while standing
	Urinate	A visible urinary stream
Locomotion	Graze	Strides taken with the head down and visibly eating the bedding
	Walk	Strides taken forward or backward
Pain	Flank gesture	Turning the head while standing to look at the side of the body
	Flehmen	Extending the head forward and curling the upper lip
	Kick	Lifting the hind leg to strike at the abdomen
	Stretch	A wide-based stance with the back ventroflexed
Resting	Stand	No movement or activity while standing with attention to the environment or in a restful posture
	Rest	Sternal or lateral recumbency

Table 6.3 Four groups of objectively measured behaviours (taken from Pritchett et al. 2003)

The subjective grouping of these behaviours makes it difficult to determine exactly what changes occurred in association with pain. Results found increased active behaviour in anaesthetised controls, which could be due to increased feeding, investigating, defecating etc. Important changes in occurring, for example a post-operative increase in sternal recumbency, may be masked by a decrease in standing. Presenting results as individual behaviours (as is the case in the current studies) allows clarification of where changes are occurring, permitting refinement of protocols to include only key behaviours, hence improving efficacy of the assessment.

6.3.5 State and event behaviour

The current studies examined *duration* of *state* behaviour and *frequency* of *event* behaviour (see section 2.2.3). The use of different methods of measuring behaviour presents problems when considering the most appropriate method for the combination of parameters to form a 'score'. However, the importance of distinguishing between state and event behaviours is highlighted by Pritchett et al. (2003). In this thesis, the duration (percentage of time spent...) of a number of behaviours were recorded and behaviours assigned to one of five groups (see table 6.2). The authors conclude that 'pain behaviour' occurs following exploratory celiotomy, but only for a small percentage of the time. The behaviours included in the 'pain behaviour' group, are all 'event' behaviours, i.e. are behavioural patterns of relatively short duration which may be saliently recorded as frequency of performance (Martin & Bateson 1993). In comparison to the 'resting behaviour' group, 'pain behaviour' contains only event behaviour, whereas 'resting behaviour' contains only state behaviour. It is therefore appears that pain behaviour only occurs for small proportion of the time. The recording of such brief events may also be inaccurate, for example, when a behaviour is recorded in seconds but only occurs for 2-3 seconds, the observer may not be able to time the behaviour accurately and the frequency of occurrence would be a more useful measure. The measurement of the duration of the events in this case, however, allows easier comparison of activity budgets for resting, activity, pain etc, which is complicated when comparing changes in frequency and duration data.

6.3.6 Summary

Detailed, objective assessments of behaviour have been used to assess pain in farm, laboratory and companion animals, removing subjective bias. In the studies reported here, duration and frequency of a wide range of behaviours were recorded with the aim of removing the influence of subjective assumptions of what may constitute 'pain behaviour' in the horse and the importance of particular behaviour for the determination of pain severity. It is accepted that these methods are labour intensive and long-term monitoring of behaviour may not be feasible for clinical pain assessment. However, the objective characterisation of potential behavioural indicators of pain, clearly defining the effects of drug protocols and hospitalisation, should form the first step in the process

of developing and validating assessment tools for clinical use. The development of clinical pain assessment tools is discussed further in section 6.5.

6.4 METHODOLOGICAL ISSUES

6.4.1 Individual variation

These studies have highlighted significant variation in the performance of behaviour. This is an issue highlighted by a number of other researchers in the field of pain behaviour. For instance Wright-Williams et al. (2007) noted significant variation in pain behaviour between different strains of laboratory mice. When considering an equine population, it is impossible to study genetically identical animals and very difficult to accurately control breed in a similar manner to that used in farm animals (Archer et al. 2004). Unlike animals such as lambs, which are reared in large groups and will generally experience similar husbandry and management techniques, young horses are not frequently 'farmed' in the same manner and will therefore be subject to differences in management, husbandry, training and experience of human beings. All of these factors may contribute to the individual variation in behaviour seen here and in order to accommodate the effects of this individual variation the number of experimental animals included in each group should be increased. However, the resultant practical, financial and time constraints might not allow for such changes. Additionally, since the variation seen here is commonly found under clinical conditions, its accommodation is considered necessary in any applied study of pain assessment.

6.4.2 Diurnal variation

Diurnal variation has a significant effect on physiological and behavioural parameters (Larsson et al. 1979; Boyd et al. 1988). The studies reported here aimed to control for the effects of diurnal variation with the following techniques;

1. Examination of treatment and control animals at the same time of day (acute model).
2. Performance of castration/sham castration at the same time of day (acute model).
3. Selection of time points for study at different times of day (chronic model).

Whilst these methods aimed to control for the effects of diurnal variation within the current studies, the number of sample points studied in both models was insufficient to determine the overall effects of diurnal variation on normal and pain-related behaviour. Therefore, care must be taken when relating the behavioural changes identified here to other horses assessed at different times of day.

6.4.3 Statistical analysis

6.4.3.1 Modelling strategies

In the work reported here a one-way ANOVA form was used, where each behavioural variable was considered separately. This approach was taken as in this preliminary work it was considered important that the changes in each individual behavioural parameter in association with pain, anaesthesia, time and time of day were fully understood. However, an alternative strategy would be to use a multivariate approach, whereby a number of behavioural variables are considered together. Testing and refining this model through the stepwise removal of parameters could then determine the subset of behavioural variables which accounted for the most variation between experimental groups (such as laminitic and control horses). This multivariate modelling approach may be valuable in development of an efficient 'tool' for the assessment of equine pain and should be considered in any future work (see section 6.5).

6.4.3.2 Cross- validation of discriminant Analysis

The use of discriminant analysis in the studies reported here aimed to identify the group of behaviours which most accurately distinguished between experimental groups. The technique provided useful information on importance of individual behaviours in the identification of pain. However, a number of flaws in the methodology used have been noted. Cross-validation should be used when performing discriminant analyses. Cross-validation can be achieved through 'leave-one-out' techniques and splitting the dataset into two subsets, performing the analysis on one subset, and then checking that the classification given is accurate for the second subset. 'Cross-validation was not performed on the analysis reported here due to the small sample size used. In future work, however, cross-validation should be incorporated into experimental design when discriminant analysis is to be used.

Exploration of other techniques for multivariate analysis (such as that mentioned in section 6.4.3.1) will be vital if a composite behavioural pain assessment tool is to be developed efficiently and objectively. Future work should consider discriminant analysis where there is a possibility for cross-validation, principal component analysis and multivariate ANOVA models.

6.4.4 Multiple Comparisons

In the studies reported here, $P<0.05$ was taken to represent statistical significance, meaning that 5% of the statistically significant results may have actually occurred by chance, therefore representing a type one error or 'false-positive' (Crawley 2002). In experimental situations such as this, where a large number of variables (behaviours in this case) from a single dataset are tested for significant associations, the number of 'false-positive' results may be relatively high. One possible approach to reduce the probability of a type one error is the use of a more conservative significance level (Rothman 1990) such as 0.01 or 0.001 or to apply specific modification such as Bonfferoni corrections. However, there is some debate within statistical literature as to whether such modifications are always appropriate (Rothman 1990). Applying a more conservative significance level may result in the generation of a type two or 'false negative error (Crawley 2002) which may mean that sensitive indices are missed.

The current studies aimed to objectively identify behavioural changes associated with pain through the examination of a wide range of behavioural variables. This design aimed to remove the influence of subjective assumptions when selecting variables to assess. These studies, did not have a series of well defined *A Priori* hypotheses. As a consequence, numerous statistical tests were performed and the approach adopted was to apply considerable caution to the interpretation of any statistically significant results. This was particularly the case with regard to the biological/clinical significance of findings rather than the absolute statistical significance. In fact, when considering all behaviours tested within general analysis, 37% of statistically significant results have a P value <0.0001 and 36% have a P value<0.01, which gives a strong indication that statistical significances seen are reflecting an actual difference.

Following on from this study, it will now be possible to frame a series of well defined hypotheses, and the consideration of modification of statistical significance levels could be considered.

6.5 FURTHER WORK

The current studies aimed to identify potential behavioural indicators of acute and chronic pain in the horse. However, if this information is to be used in a clinical setting, a system for assessment must be prepared that is not only reliable, repeatable, representative and sensitive to alterations in pain severity, but also practical for use in a clinical setting. In order for a clinically practical equine pain assessment protocol to be developed further work is required, including further analysis of the datasets described in this thesis and additional experimental testing and refinement.

1) Potential behaviours for inclusion and combination of behaviours into a clinically practical measuring 'tool'

As discussed in section 6.3, the formation of a composite scoring system based on evidence rather than assumption, is difficult to achieve.

The methodology presented here initially used a one-way ANOVA, univariate analysis of individual behavioural variables to identify potential indicators of equine pain. This provided a list of behaviours which could potentially be included in a composite score of some form. Further work should initially determine a conclusive, accurate yet efficient list of behaviours for inclusion in a composite 'tool' or score. Multivariate analysis (discriminant and tree-based model analysis) aimed to identify potential *groups* of behaviours, however, other multivariate techniques such as principle component analysis or those discussed in section 6.4.3.1 may provide additional information.

Secondly, work should address the problems encountered when combining behaviours that are measured in fundamentally different ways, for instance state behaviour measured as duration and event behaviour measured as a frequency.

It may be that the most effective 'tool' does not involve a composite type score but a general assessment of behaviour. The most effective 'tool' may not use objective measures of behaviour but rely on subjective assessment of objectively defined and validated parameters.

2) Weighting of behaviours within the measuring 'tool'

In the studies reported here tree-based model analysis aimed to determine relative importance of specific behaviours in the discrimination between 'pain' and 'no pain'. Whilst these results give some indication of relative importance of individual behaviours further work is required to develop protocols for accurately weighting behaviours within an assessment 'tool' and to validate these weightings. Behavioural weightings may vary significantly with type and location of pain, therefore requiring careful testing to determine transferability.

3) Identification of optimal duration of observation

The data presented here are based on sample periods of one hour duration. In a clinical setting, however, it would be impractical for observations of this duration to be carried out. Determination of the minimum duration of observation required to accurately detect differences between painful and pain-free horses will be necessary for the future development of a clinically viable pain assessment protocol. Further analysis is underway, using the data reported here, to reduce the duration of observation. Using The Observer™ software, the current 1-hour duration sample periods have been broken down into sample periods of 30, 15, 10, 5 and 1 minute duration. The data from these samples is being compared directly to the original data to determine whether or not the findings are similar and therefore if shorter sample periods would be adequate. Currently this analysis is univariate and based on individual behavioural parameters. The minimum observation time may, however, be reduced by the inclusion of a number of behavioural parameters in the assessment. Duration of observation should be tested following the stages outlined above.

4) Validation of sensitivity of behavioural variables as indicators of pain severity.

The current work focuses on the ability of behavioural parameters to distinguish between 'painful' and 'pain-free' horses. However, no attempt has been made to study changes in behaviour in association with pain severity. Work of this nature would not only improve the accuracy of pain assessment but act to validate behavioural changes as sensitive indicators of equine pain (Molony et al. 2002).

5) Transferability to other types of pain

This work considers only two models of pain in the horse. Further examination of models of acute pain, such as post-operative arthroscopy and chronic pain, such as osteoarthritis, is important to determine the applicability of the behavioural indicators of pain identified here to a wider range of conditions.

6.6 CONCLUSION

The general aims of the studies reported here were as follows;

1. To objectively identify behavioural biomarkers of acute and chronic pain states in the horse.
2. To determine the effects of sedative, anaesthetic and analgesic drugs on equine behaviour
3. To clarify the effects of external factors such as diurnal variation on pain-related behaviour.
4. To determine if phases of pain and no pain occur and describe their intensity and frequency.
5. To determine the most important indices for assessment of acute and chronic pain and to eliminate redundant indices, to assist in the future generation of a succinct and efficient assessment protocol.

In conclusion, behavioural indicators of both acute and chronic pain have been identified, through the objective examination of a wide range of behaviours. Pain-state specific and pain-state general behaviours highlighted and, interestingly, show the importance of different techniques for behavioural pain assessment for different pain-

states. A number of behavioural effects of standing surgical anaesthesia (or sedation) and general anaesthesia have been found. These findings are of great importance for the future accuracy of behavioural pain assessment in the horse. Additionally, diurnal variation has been shown to significantly affect behaviour and therefore identified as a key area for future study to clarify influences on pain assessment protocols. The use of Discriminant analysis and tree-based models attempted to determine the importance of behavioural variables in the assessment of pain, therefore reducing the number of behaviours observed in future work.

The studies reported here provide a detailed, objectively examination of a wide range of behavioural parameters and the effects of acute and chronic pain, hospitalisation, anaesthetic and analgesic protocols on these behaviours. This work has identified key behavioural biomarkers of pain without the influence of subjective assumptions and biases. This work provides an objective platform on which further work, developing and validating a clinically practical tool for pain assessment in horses, can be based, undoubtedly allowing wide range improvements in equine welfare.

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APPENDIX 1
BEHAVIOURAL ETHOGRAMS

A1.1 UNDISTURBED SPONTANEOUS STATE BEHAVIOUR

A1.1.1 Attentive Behaviours

Resting	Horse's body outline showing a minimum of excitement. Eyes are half closed or closed.
Attentive	Horse is in vigilant body posture and eyes completely open.
Sleeping	Horse standing or recumbent and dormant. Eyes closed.
Exploring	Horse actively investigates environment, smelling, pushing with nose at bedding, bars etc.

A1.1.2 Locomotive Behaviours

Standing	Bearing weight equally on all four limbs.
Resting forelimb	Bearing majority of weight on hindlimbs and one forelimb, whilst resting the other hindlimb.
Resting hindlimb	Bearing majority of weight on forelimbs and one hindlimb, whilst resting the other hindlimb.
Walking forwards	Horse travels forwards without obviously investigating its environment.
Walking backwards	Horse travels backwards without obviously investigating its environment.
Lateral recumbency	Lying on side with limbs extended and head and neck on substrate.
Sternal recumbency	Lying ventrally with limbs flexed.

A1.1.3 Head position

Head up	Horse's head positioned above the height of the withers.
Head down	Horse's head positioned below the height of the withers.
Head level	Horse's head positioned level wit the withers.

A1.1.4 Ear position

Forward	Horse's ears are orientated towards the front.
Backwards	Horse's ears are orientated towards the back.
Sidewards	Horse's ears are orientated towards the side.

A1.1.5 Lip position

Lip tense	Horse's lower lip is tightly wrinkled and tense.
Lip relaxed	Horse's lower lip is loose and may be sagging slightly open.

A1.1.6 Tail position

Tail relaxed	Tail lies loosely over the rump.
Tail raised	Tail is held in an elevated position.
Tail depressed	Dock of tail is pressed tightly against rump.

A1.1.7 Position in the box

Front of box	Horse is positioned in the front (nearest the door) ⅓ of the stable.
Back of box	Horse is positioned in the back (furthest from door) ⅓ of the stable.
Middle of box	Horse is positioned in the middle ⅓ of the stable.

A1.1.8 Oral Behaviour

Eating forage	Horse ingests forage (hay or haylage).
Eating concentrate	Horse ingests concentrate food.
Eating bedding	Horse ingests bedding material.
Drinking	Horse ingests water from the bucket or automatic drinker.
Grooming	Horse moves tongue or teeth over it's body, biting or licking the pelage.
Biting	Horse moves mouth over an object (bucket, person, wall, bars etc), directing incisors at surface.
Licking	Movement of tongue on object such as bucket, wall or bars.
Smelling	Movement of muzzle towards object, followed by inhalation.
Tooth Grinding	Repetitive movements of lower jaw causing friction between upper and lower molars. Associated with grinding sound.
Licking & Chewing	Horse licks and chews repetitively in the absence of any food in mouth.

A.1.1.9 Stereotypical Behaviour

Weaving	Swinging head from side to side while shifting weight alternatively between both forelimbs.
Stall-kicking	Horse kicks the door or sides of the stable.
Crib-biting	Horse uses teeth to take hold of some form of projection and sucks in air.
Wind-sucking	Horse arches neck and swallows air without clamping teeth onto any projection.
Box walking	Repetitive walking of circle around stable.
Head pressing	Leaning forehead against a vertical surface

A1.1.10 Other

Flared nostrils	Maximally dilated nostrils.
Sweating	Secretion of moisture from skin, which dampens pelage.

A1.2 UNDISTURBED SPONTANEOUS EVENT BEHAVIOUR

A1.2.1 Locomotive Behaviours

Kicking	Horse suddenly flexes and elevates hindlimb(s), thrust quickly posteriorly.
Forelimb stamping	Horse raises and forcefully lowers forelimb onto substrate.
Hindlimb stamping	Horse raises and forcefully lowers hindlimb onto substrate.
Forelimb lifting	Horse raises and lowers forelimb in less than 3 seconds
Hindlimb lifting	Horse raises and lowers hindlimb in less than 3 seconds.
Weight-shifting	Temporary weight bearing on all four limbs, followed by relaxation in one limb, causing weight to be distributed over the remaining three limbs.
Striking	Horse makes swift motion with one or both forelimbs in an anterior direction.
Pawing	Dragging toe posteriorly in a digging or scraping motion.
Scratching	Horse extends hindlimb so that hoof rubs lowered head or neck.
Pushing	Horse uses part of body to press against something in an attempt to displace it.
Rolling	Rotation on to the back, flexing limbs while recumbent.
Rubbing	Horse moves lower jaw surface against its forearm or horse moves any part of its body, back and forth or up and down against any object.
Stretch To Urinate	Hindlimbs extended posteriorly (weight on toes) with back stretched in braced stance.
Shaking	Surface of body as well as head and neck are rotated or vibrated rapidly.
Kicking Abdomen	Anterior motion of hindlimb towards abdomen.
Lying Down	Horse goes from standing position to sternal recumbency.
Getting Up	Horse goes from sternal recumbency to standing position.
Buck	Horse suddenly flexes and elevates both hind limbs, thrust quickly posteriorly as the weight is shifted over the forelimbs.
Rear	Horse 'stands' up on hindlimbs, raising both forelimbs.
Hindlimb Stretch	Hindlimb posteriorly fully extended.

A1.2.2 Head and Ear Movement

Ear Flicking	Ear twitch rapidly.
Wobbling Head	Without moving the neck, horse discretely rocks the head

	horizontally in an arc-like shape.
Head Tossing	Up and down movement of neck with head flexion and extension involved.
Head Turn (Abdomen)	Movement of head and neck towards abdomen.
Head Turn (Injury)	Movement of head and neck towards injury.
Head Nod	Movement of head up and down.
Head Shake	Horse rigorously rotates both head and neck.

A1.2.3 Oral Behaviours

Bite threat	Extending head and neck while opening mouth and directing incisors at an object or person.
Snapping	Up and down movement of the jaw while lips are retracted at the corners of the mouth.
Flehmen	Head is elevated and the upper lip is raised, wrinkling the nose and exposing the gums.
Yawn	Mouth open, head extended and raised, eyes roll and close, lower jaw movement before closing mouth.

A1.2.4 Other

Urinate	Horse excretes urine.
Defecate	Horse excretes faeces.
Skin Twitch	Localised quivering of the skin.

A1.3 EVOKED STATE BEHAVIOUR

A1.3.1 Attentive Behaviours

Resting	Horse's body outline showing a minimum of excitement. Eyes are half closed or closed.
Attentive	Horse is in vigilant body posture and eyes completely open.
Sleeping	Horse standing or recumbent and dormant. Eyes closed.
Exploring	Horse actively investigates handler, smelling, pushing with nose.

A1.3.2 Locomotive Behaviours

Standing	Bearing weight equally on all four limbs.
Resting forelimb	Bearing majority of weight on hindlimbs and one forelimb, whilst resting the other hindlimb.
Resting hindlimb	Bearing majority of weight on forelimbs and one hindlimb, whilst resting the other hindlimb.

A1.3.3 Head Position

Head up (straight)	Horse's head positioned above the height of the withers and straight inline with the body.
Head down (straight)	Horse's head positioned below the height of the withers and straight inline with the body.
Head level (straight)	Horse's head positioned level wit the withers and straight inline with the body.
Head up (turned towards handler)	Horse's head positioned above the height of the withers and turned in the direction of the handler (left hand side).
Head down (turned towards handler)	Horse's head positioned below the height of the withers and turned in the direction of the handler (left hand side).
Head level (turned towards handler)	Horse's head positioned level wit the withers and turned in the direction of the handler (left hand side).
Head up (turned away from handler)	Horse's head positioned level wit the withers and turned away from the handler (right hand side).
Head down (turned away from handler)	Horse's head positioned below the height of the withers and turned away from the handler (right hand side).
Head level (turned away from handler)	Horse's head positioned level wit the withers and turned away from the handler (right hand side).

A.1.3.4 Ear Position

Forward	Horse's ears are orientated towards the front.
Backwards	Horse's ears are orientated towards the back.
Sidewards	Horse's ears are orientated towards the side.

A1.3.5 Tail Position

Tail relaxed	Tail lies loosely over the rump.
Tail raised	Tail is held in an elevated position.
Tail depressed	Dock of tail is pressed tightly against rump.

A1.3.6 Head and Ear Movement

Ear Flicking	Ear twitch rapidly.
Wobbling Head	Without moving the neck, horse discretely rocks the head horizontally in an arc-like shape.
Head Tossing	Up and down movement of neck with head flexion and extension involved.
Head Turn (Abdomen)	Movement of head and neck towards abdomen.
Head Turn (Injury)	Movement of head and neck towards injury.
Head Nod	Movement of head up and down.
Head Shake	Horse rigorously rotates both head and neck.

A1.3.7 Oral Behaviours

Biting	Horse moves mouth over handler, directing incisors at handler
Licking	Movement of tongue on handler.
Smelling	Movement of muzzle towards handler, followed by inhalation.
Tooth Grinding	Repetitive movements of lower jaw causing friction between upper and lower molars. Associated with grinding sound.
Licking & Chewing	Horse licks and chews repetitively in the absence of any food in mouth.

A1.3.8 Other Behaviours

Flared nostrils	Maximally dilated nostrils.
Sweating	Secretion of moisture from skin, which dampens pelage.

A1.4 EVOKED EVENT BEHAVIOUR

A1.4.1 Locomotive Behaviours

Stepping away	Horses raises and lowers fore or hindlimb resulting in movement away from the handler.
Kicking	Horse suddenly flexes and elevates hindlimb(s), thrust quickly posteriorly.
Forelimb stamping	Horse raises and forcefully lowers forelimb onto substrate.
Hindlimb stamping	Horse raises and forcefully lowers hindlimb onto substrate.
Forelimb lifting	Horse raises and lowers forelimb in less than 3 seconds
Hindlimb lifting	Horse raises and lowers hindlimb in less than 3 seconds
Weight-shifting	Temporary weight bearing on all four limbs, followed by relaxation in one limb, causing weight to be distributed over the remaining three limbs.
Striking	Horse makes swift motion with one or both forelimbs in an anterior direction.
Pawing	Dragging toe posteriorly in a digging or scraping motion.
Shaking	Surface of body as well as head and neck are rotated or vibrated rapidly.

A1.4.2 Oral Behaviours

Bite threat	Extending head and neck while opening mouth and directing incisors at an object or person.
Snapping	Up and down movement of the jaw while lips are retracted at the corners of the mouth.
Flehmen	Head is elevated and the upper lip is raised, wrinkling the nose and exposing the gums.
Yawn	Mouth open, head extended and raised, eyes roll and close, lower jaw movement before closing mouth.

A1.4.3 Other Behaviours

Urinate	Horse excretes urine.
Defecate	Horse excretes faeces.
Skin Twitch	Localised quivering of the skin.

APPENDIX TWO

CHAPTER THREE RESULTS

A2. 1 HORSE DETAILS

Experimental Group	Horse Number	Age	Breed	Vet	Stable (w x d)	Stable size
Castrate	1	2	Thoroughbred	1	3m x 4.2m	12.6 m^2
Castrate	2	3	Thoroughbred	2	4.5m x 4.5m	20.25 m^2
Castrate	3	3	Thoroughbred	2	5m x 4.8m	24 m^2
Castrate	4	2	Thoroughbred	1	3m x 3.5m	10.5 m^2
Castrate	5	2	Thoroughbred	1	2m x 3m	6 m^2
Castrate	6	2	Thoroughbred	3	5.8m x 2.5m	14.5 m^2
Castrate	7	2	Thoroughbred	1	4mx4m	16 m^2
Castrate	8	3	Thoroughbred	1	4mx4m	16 m^2
Castrate	9	3	Thoroughbred	1	4mx4m	16 m^2
Castrate	10	3	Thoroughbred	2	3.5m x 3.5m	12.25 m^2
Control	1	3	Thoroughbred		4m x 5m	20 m^2
Control	2	3	Thoroughbred		4m x 5m	20 m^2
Control	3	3	Thoroughbred		4m x 5m	20 m^2
Control	4	3	Thoroughbred		4m x 5m	20 m^2
Control	5	6	Warmblood		4m x 5m	20 m^2
Control	6	5	Warmblood		4m x 5m	20 m^2
Control	7	5	Thoroughbred		4m x 5m	20 m^2
Control	8	1	Thoroughbred		4m x 5m	20 m^2
Control	9	2	TBxWarmblood		4m x 5m	20 m^2
Control	10	3	Warmblood		4m x 5m	20 m^2

Table A1.1 Details of experimental animals

A2.2 SPONTANEOUS BEHAVIOUR RESULTS

Behaviour	Univariate Statistics			Multivariate Statistics	
	Experimental Group	Treatment	Time	Interaction Group*Treatment	Interaction Group*Time
Inattentive (rest hind)	T_{17}=-1.2, P=0.248	T_{346}=2.81, **P=0.005**	T_{346}=-0.69, P=0.491	T_{345}=-1.81, P=0.071	T_{245}=-0.75, P= 0.457
Total lying	T_{17}=-1.42, 0.174	T_{346}=-0.93, P=0.354	T_{346}=0.57, P=0.571	T_{345}=0.29, P=0.774	T_{345}=0.14, P=0.885
Head low (adj. feed)	T_{17}=-2.9, **P=0.009**	T_{346}=6.1, **P<0.001**	T_{346}=-1.6, P=0.111	T_{345}=-3.7, **P<0.001**	T_{345}=0.4, P=0.703
Exploratory	T_{17}=-6.78 **P<0.001**	T_{346}=1.79, P=0.075	T_{346}=0.17, P=0.864	T_{345}=0.30, P=0.864	T_{345}=-0.48, P=0.761
Stand	T_{17}=-0.29, P=0.779	T_{346}=0.45, P=0.653	T_{346}=0.70, P=0.703	T_{345}=0.63, P=0.527	T_{345}=1.51 P=0.132
Rest hindlimb	T_{17}=0.97, P=0.344	T_{346}=0.0, P=0.900	T_{346}=-0.89, P=0.375	T_{345}=-1.52, P=0.128	T_{345}=-1.92, P=0.056
Walk	T_{17}=1.59, P=0.135	T_{346}=0.9, P=0.369	T_{346}=-0.08, P=0.932	T_{345}=1.08, P=0.281	T_{345}=0.65, P=0.517
Lie sternally	T_{17}=-1.01, P=0.325	T_{346}=-1.06, P=0.290	T_{346}=0.78, P=0.433	T_{345}=0.88, P=0.378	T_{345}=-0.03, P=0.974
Lie laterally	T_{17}=-1.83, P=0.085	T_{346}=-0.23 P=0.815	T_{346}=-0.11, P=0.913	T_{345}=-1.05, P=0.292	T_{345}=0.32, P=0.746
Grooming	T_{17}=-0.16, P=0.874	T_{346}=2, **P=0.049**	T_{346}=0.00 P=0.998	T_{345}=0.46, P=0.646	T_{345}=3.78, **P<0.001**
Head up(adj.feed)	T_{17}=2.9, **P=0.01**	T_{346}=-6.1, **P<0.001**	T_{346}=1.6, P=0.111	T_{345}=3.7, **P<0.001**	T_{345}=-0.4, P=0.704
Head down (adj.feed)	T_{17}=-0.2, P=0.847	T_{346}=4.4, **P<0.001**	T_{346}=-1.2, P=0.232	T_{346}=-1.2, P=0.247	T_{345}=0.7, P=0.476
Head Level (adj.feed)	T_{17}=-3.0, **P=0.008**	T_{346}=4.9, **P<0.001**	T_{346}=-1.3, P=0.184	T_{345}=-3.5, **P<0.001**	T_{345}=0.0, P=0.973
Front	T_{17}=6.03, **P<0.001**	T_{346}=2.25, **P=0.032**	T_{346}=0.0, P=0.999	T_{345}=0.08, P=0.933	T_{345}=2.07, **P=0.039**
Middle	T_{17}=-2.22, **P=0.041**	T_{346}=-1.25, P=0.212	T_{346}=1.32 P=0.185	T_{345}=-1.20, P=0.194	T_{345}=-0.53, P=0.595
Back	T_{17}=-3.92, **P=0.001**	T_{346}=-0.65, P=0.118	T_{346}=-0.85, P=0.394	T_{345}=0.38, P=0.698	T_{345}=-1.90, P=0.059

Table A2.2.1 Results of general (overall) analysis for state behaviours using univariate (the effect of a single variable is considered) and multivariate (the effect of a number of variables and the interaction between these variables is considered) statistics. Time = hours post-intervention and equivalent baseline time points, experimental group = castrate or control, treatment = baseline or post-intervention.

Behaviour	Univariate Statistics			Multivariate Statistics	
	Experimental Group	Treatment	Time	Group*Treatment	Group*Time
Leg movement	$F_{1,17}$=0.2, P=0.697	$F_{1,346}$=1.7, P=0.199	$F_{1,346}$=0.0, P=0.961	$F_{1,345}$=0.9, P=0.357	$F_{1,345}$=6.3, **P=0.013**
Weight Shift	$F_{1,17}$=0.0, P=0.851	$F_{1,346}$=4.5, **P=0.035**	$F_{1,346}$=0.0, P=0.843	$F_{1,345}$=0.0, P=0.906	$F_{1,345}$=8.6, **P=0.004**
Lift Forelimb	$F_{1,17}$=0.5, P=0.504	$F_{1,346}$=0.1, P=0.803	$F_{1,17}$=0.0, P=0.574	$F_{1,345}$=0.2, P=0.670	$F_{1,345}$=1.8, P=0.181

Table A2.2.2 Results of general (overall) analysis for event behaviours using univariate (the effect of a single variable is considered) and multivariate (the effect of a number of variables and the interaction between these variables is considered) statistics. Time = hours post-intervention and equivalent baseline times, experimental group = castrate or control, treatment = baseline or post-intervention.

Behaviour	*Is there a difference in whether it happens or not?*				
	Univariate Statistics			Multivariate Statistics	
	Experimental Group	Treatment	Time	Group*Treatment	Group*Time
Paw	T_{17}=2.0, P=0.061	T_{346}=-0.2, P=0.831	T_{346}=-0.5, P=0.728	T_{345}=-0.1, P=0.958	T_{345}=-2.4, **P=0.018**
Stamp	T_{17}=2.0, P=0.066	T_{346}=-5.3, **P<0.001**	T_{346}=0.6, P=0.558	T_{345}=0.0, P=0.999	T_{345}=-2.0, P=0.051
Lift Hindlimb	T_{17}=1.4 P=0.167	T_{346}=-0.8, P=0.448	T_{346}=-0.7, P=0.467	T_{345}=1.2, P=0.218	T_{345}=0.7, P=0.463
Kick	T_{17}0.0, P=0.999	T_{346}=-0.0, P=0.999	T_{346}=0.0, P=0.963	T_{345}=0.0, P=0.999	T_{345}=0.0, P=0.999
Tail Flick	T_{17}=-1.3, P=0.223	T_{346}=0.4, P=0.677	T_{346}=0.5, P=0.590	T_{345}=2.2, **P=0.030**	T_{345}=-0.3, P=0.743
Head Shake	T_{17}=1.81, P=0.087	T_{346}=0.37, P=0.706	T_{346}=-1.08, P=0.282	T_{346}=-1.80, P=0.073	T_{345}=0.04, P=0.966

Table A2.2.3 Results of occurrence analysis for event behaviours for which square root transformation was insufficient to normalise the distribution of residuals, due to high numbers of zero values (no behaviour performed in sample).

Behaviour	*If it does happen, is there a difference in how much?*				
	Univariate Statistics			**Multivariate Statistics**	
	Experimental Group	**Treatment**	**Time**	**Group*Treatment**	**Group*Time**
Paw	$F_{1,7}$=1.3, P=0.264	$F_{1,65}$=0.8, P=0.376	$F_{1,65}$=3.3, P=0.137	$F_{1,64}$=0.1, P=0.745	$F_{1,64}$=0.6, P=0.451
Stamp	$F_{1,6}$=0.4, P=0.542	$F_{1,16}$=0.4, P=0.533	$F_{1,16}$=0.0, P=0.909	NA	$F_{1,15}$=0.6, P=0.460
Lift Hindlimb	$F_{1,17}$=4.1, P=0.058	$F_{1,151}$=9.7, **P=0.002**	$F_{1,151}$=1.5, P=0.223	$F_{1,150}$=3.2, P=0.075	$F_{1,150}$=2.0, P=0.158
Kick	NA	NA	NA	NA	NA
Tail Flick	$F_{1,,17}$=1.7, P=0.207	$F_{1,167}$=2.9, P=0.091	$F_{1,167}$=0.2, P=0.682	$F_{1,166}$=0.0, P=0.929	$F_{1,166}$=0.8, P=0.375
Head Shake	$F_{1,16}$=0.0, P=0.927	$F_{1,108}$=0.8, P=0.370	$F_{1,108}$=6.0, **P=0.016**	$F_{1,107}$=0.0, P=0.945	$F_{1,107}$=0.2, P=0.673

Table A2.2.4 Results of level of occurrence analysis for event behaviours for which square root transformation was insufficient to normalise the distribution of residuals, due to high numbers of zero values (no behaviour performed in sample).'NA' indicates samples where insufficient data precluded statistical testing, due to lack of performance of behaviour.

A2.3 TIME POINT ANALYSIS RESULTS

Behaviour	Time post-intervention		
	6 hours	16 hours	20 hours
Inattentive (rest hind)	W=38.5, P=0.856	W=39.5, P=0.964	W=52, P=0.335
Total lying	W=45, P=0.374	W=49, P=0.450	W=52, P=0.627
Head low (adj. feed)	W=62, P=0.062	W=59, P=0.112	W=62.5, P=0.165
Exploratory	W=52, P=0.327	W=56.5, P=0.143	W=60, P=0.230
Stand	W=29, P=0.331	W=35, P=0.659	W=61.5, P=0.191
Rest hindlimb	W=47, P=0.6048	W=32, P=0.480	W=25.5, P=0.121
Walk	W=31, P=0.424	W=34, P=0.707	W=60, P=0.224
Lie sternally	W=45, P=0.374	W=43, P=0.850	W=50.5, P=0.659
Lie laterally	NA	W=58.5, **P=0.034**	W=52.5, P=0.509
Grooming	W=31.5, P=0.169	W=49.5, P=0.169	W=40.5, P=0.399
Head up(adj.feed)	W=49.5, P=0.452	W=22, P=0.112	W=14, **P=0.013**
Head down (adj.feed)	W=55, P=.197	W=31.5, P=0.438	W=49.5, P=0.732
Head Level (adj.feed)	W=57.5, P=0.141	W=67, **P=0.021**	W=54.5, P=0.461
Front	W=30, P=0.374	W=14.5, **P=0.023**	W=29.5, P=0.218
Middle	W=64, **P=0.026**	W=67, P=0.135	W=57, P=0.321
Back	W=49, P=0.478	W=54, P=0.25	W=65, P=0.108
Leg move	W=61, P=0.076	W=34.5, P=0.627	W=32, P=0.306
Weight shift	W=52, P=0.387	W=25.5, P=0.199	W=31, P=0.269
Stamp	W=49.5, P=0.184	W=45, P=0.374	W=50, P=0.343
Paw	W=58.5, P=0.096	W=40.5, P=0.999	W=41, P=0.651
Kick	W=49.5, P=0.169	NA	W=50, P=0.343
Lift fore limb	W=53.5, P=0.268	W=34.5, P=0.626	W=45, P=0.999
Lift Hind limb	W=63, **P=0.027**	W=56.5, P=0.149	W=36, P=0.468
Head shake	W=38.5, P=0.8557	W=44, P=0.684	W=41, P=0.740
Tail flick	W=45, P=0.587	W=33.5, P=0.527	W=42, P=0.832

Table A2.3.1 Comparison of castrate and control horses at 6, 16, and 20 hours post-intervention using Wilcoxon rank sum test. 'NA' indicates samples where insufficient data precluded statistical testing, due to lack of performance of behaviour.

Behaviour	Time post-intervention		
	6 hours	16 hours	20 hours
Inattentive	V=5.5 , P=0.999	V=4, P=0.208	V=3, P=0.584
Total lying	V=0, P=0.999	V=24, P=0.441	V=3, P=0.281
Head low	V=3, P=0.042	V=6, P=0.055	V=14, P=0.359
Exploratory	V=8, P=0.183	V=10, P=0.999	V=29, P=0.476
Stand	V=28, P=0.570	V=35, P=0.164	V=15, P=0.426
Rest hindlimb	V=20, P=0.820	V=5, P=**0.040**	V=26, P=0.052
Walk	V=22, P=0.999	V=25.5, P=0.326	V=15.5, P=0.344
Lie sternally	V=0, P=0.999	V=27, P=0.234	V=1, P=0.106
Lie laterally	NA	V=9, P=0.834	V=5, P=0.999
Grooming	V=3, P=0.371	V=0, P=0.346	V=1, P=0.999
Head up	V=36, **P=0.014**	V=37, P=0.1	V=31, P=0.359
Head down	V=11, P=0.670	V=9, P=0.833	V=7, P=0.263
Head Level	V=5, P=0.08	V=6, P=0.055	V=20, P=0.820
Front	V=21, P=0.910	V=35, P=0.164	V=16, P=0.496
Middle	V=15, P=0.859	V=21, P=0.910	V=35.5, P=0.138
Back	V=24.5, P=0.859	V=10, P=0.294	V=13, P=0.301
Leg move	V=16.5, P=0.514	V=12, P=0.800	V=30, P=0.426
Weight shift	V=22.5, P=0.999	V=15, P=0.932	V=24, P=0.441
Stamp	V=0, P=0.371	V=0, P=0.999	V=0, P=0.999
Paw	V=10, P=0.560	V=1, P=0.999	V=3, P=0.371
Kick	V=0, P=0.371	NA	V=0, P=0.999
Lift forelimb	V=24, P=0.906	V=30.5, P=0.374	V=21, P=0.529
Lift Hindlimb	V=2.5, P=0.063	V=1, P=0.106	V=16, P=0.799
Head shake	V=12, P=0.281	V=10.5, P=0.480	V=23.5, P=0.125
Tail flick	V=6, P=0.854	V=13.5, P=0.136	V=3, P=0.075

Table A2.3.2 Paired comparison of baseline to post-intervention values in castrate horses at 6, 16, and 20 hours post-intervention using Wilcoxon signed rank test. 'NA' indicates samples where insufficient data precluded statistical testing, due to lack of performance of behaviour.

Behaviour	Time post-intervention		
	6 hours	16 hours	20 hours
Inattentive	V=1, P=0.423	V=6.5, P=0.462	V=3, P=0.999
Total lying	V=1, P=0.999	V=33, **P=0.042**	V=5, P=0.588
Head low	V=21, P=0.906	V=30, P=0.426	V=27, P=0.999
Exploratory	V=1.5, P=0.074	V=6.5, P=0.892	V=16, P=0.800
Stand	V=20.5, P=0.779	V=12.5, P=0.260	V=44, P=0.106
Rest hindlimb	V=20, P=0.820	V=13, P=0.286	V=15, P=0.232
Walk	V=6, P=0.107	V=1, P=0.056	V=35.5, P=0.131
Lie sternally	V=1, P=0.999	V=33, **P=0.042**	V=2, P=0.178
Lie laterally	NA	V=6, P=0.181	V=3, P=0.999
Grooming	V=0, P=0.999	NA	V=0, P=0.999
Head up	V=43, **P=0.012**	V=28, P=0.570	V=18, P=0.375
Head down	V=2, P=9.345	V=3, P=0.141	V=22, P=0.188
Head Level	V=22, P=0.999	V=41, **P=0.032**	V=17, P=0.307
Front	V=21, P=0.906	V=7, P=0.074	V=24, P=0.760
Middle	V=7, P=0.581	V=36, P=0.189	V=19, P=0.447
Back	V=17, P=0.944	V=19.5, P=0.889	V=16, P=0.773
Leg move	0.447	V=18, P=0.652	V=22, P=0.999
Weight shift	V=24, P=0.910	V=18, P=0.635	V=24, P=0.906
Stamp	NA	NA	NA
Paw	V=1, P=0.999	V=0, P=0.999	V=1.5, P=0.999
Kick	NA	NA	NA
Lift forelimb	V=17, P=0.570	V=11, P=0.203	V=31, P=0.343
Lift Hindlimb	V=7, P=0.581	V=4, P=0.408	V=1.5, P=0.074
Head shake	V=3, P=0.999	V=1, P=0.999	V=6.5, P=0.892
Tail flick	V=45, **P=0.009**	V=3.5, P=0.343	V=10, P=0.294

Table A2.3.3 Paired comparison of baseline to post-intervention values in control horses at 6, 16, and 20 hours post-intervention using Wilcoxon signed rank test. 'NA' indicates samples where insufficient data precluded statistical testing, due to lack of performance of behaviour.

A2.4 EVOKED BEHAVIOUR RESULTS

Behaviour	Univariate Statistics			Multivariate Statistics	
	Experimental Group	Treatment	Time	Group*Treatment	Group*Time
Ears back	T_{17}=-2.8, **P=0.012**	T_{163}=5.3, **P<0.001**	T_{163}=-2.2, **P=0.032**	T_{162}=-4.2, **P<0.001**	T_{162}=0.7, P=0.479
Head up (towards handler)	T_{17}=-0.7, P=0.483	T_{163}=0.0, P=0.986	T_{163}=-0.6, P=0.527	T_{162}=0.9, P=0.363	T_{162}=-0.5, P=0.602
Head up (away from handler)	T_{17}=-1.0, P=0.314	T_{163}=2.0, P=0.051	T_{163}=-0.1, P=0.954	T_{162}=-2.3, **P=0.022**	T_{162}=1.2, P=0.224
Head up - straight	T_{17}=-0.2, P=0.835	T_{163}=-1.94, P=0.054	T_{163}=1.0, P=0.340	T_{162}=-0.54, P=0.590	T_{162}=0.7 P=0.439
Head down (towards handler)	T_{17}=2.6, P=0.017	T_{163}=2.8, P=0.006	T_{163}=-0.6, P=0.528	T_{162}=-0.0, P=0.990	T_{162}=-1.0, P=0.338
Head down - straight	T_{17}=1.0, P=0.353	T_{163}=2.5, P=0.012	T_{163}=-0.7, P=0.482	T_{162}=-0.0, P=0.999	T_{162}=-0.2, P=0.866
Exploratory	T_{17}=0.3, P=0.801	T_{163}=1.3, P=0.195	T_{163}=1.3 P=0.209	T_{162}=1.3 P=0.191	T_{162}=-1.4, P=0.171

Table A2.4.1 Results of general (overall) analysis for state behaviours performed during interactive testing, using univariate (the effect of a single variable is considered) and multivariate (the effect of a number of variables and the interaction between these variables is considered).

Behaviour	Univariate Statistics			Multivariate Statistics	
	Experimental Group	Treatment	Time	Group*Treatment	Group*Time
Stamp	T_{17}=0.0, P=0.766	NA	NA	T_{161}=0.0, P=0.989	NA
Step Away	T_{17}=3.1, **P=0.007**	T_{162}=-4.1, **P<0.001**	T_{162}=2.0, **P=0.046**	T_{161}=0.0, **P<0.001**	T_{161}=-0.1, P=0.930
Tail Flick	T_{17}=1.7, P=0.099	T_{162}=-0.0, P=0.999	T_{162}=-0.8, P=0.442	T_{161}=54.9, **P<0.001**	T_{161}=0.3, P=0.763
Forelimb lift	T_{17}=0.8, P=0.428	T_{162}=0.0, P=0.999	T_{162}=-2.2, **P=0.025**	T_{161}=-0.0, P=0.999	T_{161}=3.9, **P<0.001**
Ear Flick	T_{17}=0.0, P=0.766	NA	NA	T_{161}=0.0, **P<0.001**	NA

Table A2.4.2 Results of occurrence analysis for event behaviours occurring during interactive testing, for which square root transformation was insufficient to normalise the distribution of residuals, due to high numbers of zero values (no behaviour performed in sample).

Behaviour	Univariate Statistics			Multivariate Statistics	
	Experimental Group	Treatment	Time	Group*Treatment	Group*Time
Stamp	NA	NA	NA	NA	NA
Step Away	$F_{1,9}$=0.0, P=0.931	$F_{1,1}$=1.1, P=0.323	$F_{1,8}$=0.1, P=0.755	NA	$F_{1,7}$=0.3, P=0.592
Tail Flick	$F_{1,4}$=0.6, P=0.499	NA	$F_{1,1}$=3,2, P=0.324	NA	NA
Forelimb lift	$F_{1,1}$=0.0, P=0.364	$F_{1,}$=0.0, P=0.404	NA	NA	NA
Ear Flick	NA	NA	NA	NA	NA

Table A2.4.3 Results of level of occurrence analysis for event behaviours, occurring during interactive testing for which square root transformation was insufficient to normalise the distribution of residuals, due to high numbers of zero values (no behaviour performed in sample).'NA' indicates samples where insufficient data precluded statistical testing, due to lack of performance of behaviour.

APPENDIX THREE

CHAPTER FOUR RESULTS

A3.1 HORSE DETAILS

Experimental Group	Number	Breed	Age (y,m)	Weight	Vet
Castrate	1	Thoroughbred	0,9	318	SB
Castrate	2	Thoroughbred	1,0	373	BM
Castrate	3	Arab	2,3	358	BM
Castrate	4	Thoroughbred	1,0	399	SB
Castrate	5	Thoroughbred X	1,4	402	SB
Castrate	6	Warmblood	1,2	335	SB
Castrate	7	Thoroughbred X	1,3	350	SB
Castrate	8	Thoroughbred	0,6	340	SB
Castrate	9	Thoroughbred	1,0	285	SB
Castrate	10	Warmblood	3,0	501	SB
Control	1	Thoroughbred	3	508	
Control	2	Thoroughbred	3	455	
Control	3	Thoroughbred	3	447	
Control	4	Thoroughbred	3	457	
Control	5	Thoroughbred	6	588	
Control	6	Thoroughbred	5	636	
Control	7	Thoroughbred	5	514	
Control	8	Thoroughbred	1	348	
Control	9	Warmblood	2	420	
Control	10	Thoroughbred X	3	397	

Table A3.1.1 Details of experimental animals.

A3.2 SPONTANEOUS BEHAVIOUR RESULTS

Behaviour	Univariate Statistics			Multivariate Statistics	
	Experimental Group	Treatment	Time	Group*Treatment	Group*Time
Inattentive (rest hind)	T_{17}=1.1, P=0.308	T_{359}=3.8, **P<0.001**	T_{359}=1.0, P=0.326	T_{358}=-1.0, P=0.338	T_{358}=-0.3, P=0.776
Total lying	T_{17}=-0.7, P=0.508	T_{359}=-2.1, **P=0.039**	T_{359}=2.2, **P=0.028**	T_{358}=-0.2, P=0.875	T_{358}=-0.1, P=0.939
Head low	T_{17}=0.1, P=0.955	T_{359}=4.9, **P<0.001**	T_{359}=-1.8, P=0.063	T_{358}=-0.4, P=0.659	T_{358}=0.8, P=0.403
Exploratory	T_{17}=-0.6, P=0.564	T_{359}=3.2, P=**0.002**	T_{359}=-2.5, **P=0.013**	T_{358}=-0.0, P=0.978	T_{358}=-0.9, P=0.351
Stand	T_{17}=-3.0, **P=0.008**	T_{359}=-1.3, P=0.212	T_{359}=-3.1, **P=0.002**	T_{358}=-0.5, P=0.623	T_{358}=-0.6, P=0.559
Rest hindlimb	T_{17}=3.7, **P=0.002**	T_{359}=4.1, **P<0.001**	T_{359}=1.6, P=0.107	T_{358}=0.4, P=0.679	T_{358}=0.5, P=0.636
Walk	T_{17}=-2.1, P=0.051	T_{359}=-0.8, P=0.452	T_{359}=-0.7, P=0.472	T_{358}=-1.5, P=0.126	T_{358}=0.5, P=0.608
Lie sternally	T_{17}=-1.2, P=0.244	T_{359}=-2.2, **P=0.028**	T_{359}=2.0, **P=0.041**	T_{358}=0.6, P=0.543	T_{358}=0.1, P=0.946
Lie laterally	T_{17}=0.7, P=0.487	T_{359}=-0.9, P=0.358	T_{359}=1.7, P=0.090	T_{358}=-2.1, **P=0.037**	T_{358}=-0.5, P=0.611
Grooming	T_{17}=-2.4, **P=0.026**	T_{359}=0.8, P=0.417	T_{359}=0.5, P=0.616	T_{358}=1.1, P=0.260	T_{358}=-1.6, P=0.106
Head up	T_{17}=0.1, P=0.927	T_{359}=-5.1, **P<0.001**	T_{359}=2.0, **P=0.042**	T_{358}=0.2, P=0.840	T_{358}=-0.5, P=0.616
Head down	T_{17}=-2.6, **P=0.018**	T_{359}=2.3, **P=0.023**	T_{359}=-3.9, **P<0.001**	T_{358}=-0.2, P=0.843	T_{358}=0.6, P=0.591
Head Level	T_{17}=1.8, P=0.085	T_{359}=3.5, **P<0.001**	T_{359}=0.8, P=0.431	T_{358}=-0.3, P=0.752	T_{358}=0.0, P=0.968
Front	T_{17}=1.2, P=0.236	T_{359}=-0.3, P=0.727	T_{359}=-3.2, **P<0.001**	T_{358}=-1.0, P=0.298	T_{358}=-0.2, P=0.811
Middle	T_{17}=-0.7, P=0.493	T_{359}=-0.1, P=0.890	T_{359}=0.2, P=0.864	T_{358}=0.5, P=0.635	T_{358}=1.7, P=0.095
Back	T_{17}=-0.8, P=0.463	T_{359}=0.5, P=0.621	T_{359}=3.5, **P<0.001**	T_{358}=0.8, P=0.401	T_{358}=-0.8, P=0.439

Table A3.2.1 Results of general (overall) analysis for state behaviours using univariate (the effect of a single variable is considered) and multivariate (the effect of a number of variables and the interaction between these variables is considered) statistics. Time = hours post-intervention and equivalent baseline time points, experimental group = castrate or control, treatment = baseline or post-intervention.

Behaviour	Univariate Statistics			Multivariate Statistics	
	Experimental Group	Treatment	Time	Group*Treatment	Group*Time
Hindlimb move	$F_{1,17}$=0.2, P=0.842	$F_{1,359}$=10.1, **P=0.002**	$F_{1,359}$=4.4, **P=0.038**	$F_{1,358}$=1.9, P=0.172	$F_{1,358}$=1.9, P=0.398
Weight Shift	$F_{1,17}$=0.2, P=0.636	$F_{1,359}$=14.2, **P<0.001**	$F_{1,359}$=0.2, P=0.695	$F_{1,358}$=1.1, P=0.290	$F_{1,358}$=0.5, P=0.488
Lift Forelimb	$F_{1,17}$=0.0, P=0.856	$F_{1,359}$=9.0, **P=0.003**	$F_{1,359}$=3.5 P=0.064	$F_{1,358}$=1.2, P=0.273	$F_{1,358}$=0.8, P=0.363
Lift Hindlimb	$F_{1,17}$=0.0, P=0.860	$F_{1,359}$=0.5, P=0.4619	$F_{1,359}$=5.8, **P=0.016**	$F_{1,358}$=0.6, P=0.567	$F_{1,358}$=0.2, P=0.641

Table A3.2.2 Results of general (overall) analysis for event behaviours using univariate (the effect of a single variable is considered) and multivariate (the effect of a number of variables and the interaction between these variables is considered) statistics. Time = hours post-intervention and equivalent baseline times, experimental group = castrate or control, treatment = baseline or post-intervention.

Behaviour	*Is there a difference in whether it happens or not?*				
	Univariate Statistics			Multivariate Statistics	
	Experimental Group	Treatment	Time	Group*Treatment	Group*Time
Paw	T_{17}=3.6, P=0.061	T_{359}=-0.6, P=0.831	T_{359}=0.8, P=0.728	T_{358}=2.6, P=0.958	T_{358}=0.2, P=0.018
Stamp	T_{17}=0.8, P=0.066	T_{359}=-3.1, **P<0.001**	T_{359}=1.5, P=0.558	T_{358}=1.5, P=0.999	T_{358}=0.0, **P=0.05**
Tail Flick	T_{17}=1.7, P=0.223	T_{359}=-0.7, P=0.677	T_{359}=-0.2, P=0.590	T_{358}=-0.3, **P=0.030**	T_{358}=-1.2, P=0.743
Head Shake	T_{17}=1.5, P=0.087	T_{359}=1.4, P=0.706	T_{359}=-0.3, P=0.282	T_{358}=-2.4, P=0.073	T_{358}=0.3, P=0.966

Table A3.2.3 Results of occurrence analysis for event behaviours for which square root transformation was insufficient to normalise the distribution of residuals, due to high numbers of zero values (no behaviour performed in sample).

Behaviour	*If it does happen, is there a difference in how much?*				
	Univariate Statistics			**Multivariate Statistics**	
	Group	**Treatment**	**Time**	**Group*Treatment**	**Group*Time**
Paw	$F_{1,16}$=4.5, P=0.05	$F_{1,137}$=2.5, P=0.115	$F_{1,137}$=2.0, P=0.158	$F_{1,136}$=0.3, P=0.612	$F_{1,136}$=0.5, P=0.497
Stamp	$F_{1,4}$=0.4, P=0.556	F_{18}=1.4, P=0.267	$F_{1,8}$=2.1, P=0.185	$F_{1,7}$=0.3, P=0.604	$F_{1,7}$=0.3, P=0.588
Tail Flick	$F_{1,258}$=0.7, P=0.421	$F_{1,258}$=0.5, P=0.484	$F_{1,258}$=7.0, **P=0.009**	$F_{1,257}$=2.9, P=0.087	$F_{1,257}$=12.7, **P<0.001**
Head Shake	$F_{1,17}$=1.2, P=0.280	$F_{1,233}$=2.2, P=0.138	$F_{1,233}$=7.8, **P=0.006**	$F_{1,232}$=5.6, **P=0.01**	$F_{1,232}$=0.2, P=0.639

Table A3.2.4 Results of level of occurrence analysis for event behaviours for which square root transformation was insufficient to normalise the distribution of residuals, due to high numbers of zero values (no behaviour performed in sample).'NA' indicates samples where insufficient data precluded statistical testing, due to lack of performance of behaviour.

A3.3 TIME POINT ANALYSIS RESULTS

Behaviour	Time post-intervention	
	6 hours	16 hours
Inattentive (rest hind)	W=38, P=0.856	W=50, P=0.964
Total lying	W=45, P=0.999	W=44, P=0.964
Head low	W=25, P=0.111	W=34.5, P=0.413
Exploratory	W=30.5, P=0.237	W=54, P=0.439
Stand	W=51, P=0.653	W=53, P=0.549
Rest hindlimb	W=29, P=0.204	W=32, P=0.307
Walk	W=67, P=0.071	W=66.5, P=0.080
Lie sternally	W=45, P=0.999	W=42, P=0.813
Lie laterally	W=49.5, P=0.399	W=38, P=0.540
Grooming	W=54, P=0.193	W=39.5, P=0.597
Head up	W=65, P=0.111	W=55.5, P=0.413
Head down	W=18.5, **P=0.034**	W=27.5, P=0.165
Head Level	W=43.5, P=0.935	W=51.5, P=0.624
Front	W=43, P=0.902	W=28, P=0.171
Middle	W=46, P=0.967	W=57, P=0.344
Back	W=52.5, P=0.563	W=50.5, P=0.678
Leg move	W=45.5, P=0.999	W=40, P=0.730
Weight shift	W=49.5, P=0.741	W=38, P=0.595
Stamp	W=49.5, P=0.399	W=49.5, P=0.399
Paw	W=82, **P=0.021**	W=67, **P=0.050**
Kick	W=49.5, P=0.399	W=49,.5, P=0.400
Lift fore limb	W=36.5, =0.513	W=48.5, P=0.806
Lift Hind limb	W=42.5, P=0.87	W=39.5, P=0.68
Head shake	W=56.5, P=0.339	W=39, P=0.631
Tail flick	W=54, P=0.481	W=50.5, P=0.678

Table A3.3.1 Comparison of castrate and control horses at 6, 16, and 20 hours post-intervention using Wilcoxon rank sum test. 'NA' indicates samples where insufficient data precluded statistical testing, due to lack of performance of behaviour.

Behaviour	Time post-intervention	
	6 hours	16 hours
Inattentive (rest hind)	V=0.0, P=0.371	V=5.5, P=0.093
Total lying	V=22, P=0.205	V=47, P=0.049
Head low	V=, P=0.557	V=14, P=0.193
Exploratory	V=9, P=0.787	V=9, P=0.232
Stand	V=20, P=0.492	V=17, P=0.322
Rest hindlimb	V=29.5, P=0.959	V=6, P=0.052
Walk	V=28, P=0.999	V=33, P=0.235
Lie sternally	V=22, P=0.205	V=42, **P=0.024**
Lie laterally	V=4, P=0.789	V=25, P=0.360
Grooming	V=10, P=0.572	V=6.5, P=0.710
Head up	V=34, P=0.557	V=41, P=0.193
Head down	V=20, P=0.475	V=15, P=0.221
Head Level	V=25, P=0.846	V=18, P=0.375
Front	V=22, P=0.999	V=23, P=0.909
Middle	V=31, P=0.343	V=20, P=0.813
Back	V=20, P=0.834	V=29.5, P=0.878
Leg move	V=21, P=0.557	V=20.5, P=0.507
Weight shift	V=2, P=0.999	V=11.5, P=0.213
Stamp	V=0, P=0.999	V=0, P=0.999
Paw	V=9, P=0.233	V=15.5, P=0.779
Kick	V=0, P=0.999	V=0, P=0.999
Lift fore limb	V=28, P=0.999	V=16, P=0.275
Lift Hind limb	V=26, P=0.722	V=19, P=0.944
Head shake	V=9.5, P=0.916	V=19, P=0.450
Tail flick	V=22.5, P=0.999	V=23, P=0.528

Table A3.3.2 Paired comparison of baseline to post-intervention values in castrate horses at 6, 16, and 20 hours post-intervention using Wilcoxon signed rank test. 'NA' indicates samples where insufficient data precluded statistical testing, due to lack of performance of behaviour.

Behaviour	Time post-intervention	
	6 hours	16 hours
Inattentive (rest hind)	V=17.5, P=0.172	V=2, P=0.094
Total lying	V=5, P=0.423	V=36, **P=0.014**
Head low	V=6, P=0.107	V=8, P=0.097
Exploratory	V=12, P=0.440	V=7, P=0.999
Stand	V=11, P=0.672	V=15, P=0.407
Rest hindlimb	V=21.5, P=0.674	V=3, **P=0.020**
Walk	V=4, **P=0.034**	V=9, P=0.833
Lie sternally	V=5, P=0.414	V=45, **P=0.004**
Lie laterally	V=3, P=0.371	V=28, P=0.183
Grooming	V=1, P=0.999	V=0, P=0.149
Head up	V=30, P=0.107	V=37, P=0.097
Head down	V=7, P=0.074	V=13, P=0.529
Head Level	V=13.5, P=0.575	V=6, P=0.107
Front	V=10, P=0.164	V=13, P=0.301
Middle	V=12.5, P=0.866	V=10, P=0.999
Back	V=28, P=0.183	V=23, P=0.151
Leg move	V=11, P=0.363	V=7, P=0.074
Weight shift	V=20, P=0.353	V=6, P=0.055
Stamp	V=1, P=0.999	NA
Paw	V=6, P=0.174	V=0, P=0.999
Kick	NA	NA
Lift fore limb	V=9, P=0.447	V=8, P=0.098
Lift Hind limb	V=6, P=0.402	V=11, P=0.672
Head shake	V=24.5, P=0.398	V=1, P=0.423
Tail flick	V=20, P=0.352	V=15.5, P=0.779

Table A3.3.3 Paired comparison of baseline to post-intervention values in control horses at 6, 16, and 20 hours post-intervention using Wilcoxon signed rank test. 'NA' indicates samples where insufficient data precluded statistical testing, due to lack of performance of behaviour.

A3.4 DIRECT OBSERVATION SPONTANEOUS BEHAVIOURS RESULTS

Behaviour	Univariate Statistics			Multivariate Statistics	
	Experimental Group	Treatment	Time	Group*Treatment	Group*Time
Attentive	T_{17}=-1.0, P=0.420	T_{332}=-0.4, P=0.577	T_{332}=0.2, P=0.778	T_{331}=1.1, P=0.313	T_{331}=-0.7, P=0.629
Ears forward	T_{17}=-0.5, P=0.747	T_{332}=-3.7, **P<0.001**	T_{332}=-0.8, P=0.582	T_{331}=1.8, P=0.122	T_{331}=-1.5, P=0.209
Ears back	T_{17}=-1.5, P=0.163	T_{332}=1.6, P=0.1083	T_{332}=1.8, P=0.070	T_{331}=-1.9, P=0.060	T_{331}=1.5, P=0.122
Ears side	T_{17}=3.2, **P=0.005**	T_{332}=3.3, **P<0.001**	T_{332}=-2.0, P=0.046	T_{331}=-0.8, P=0.450	T_{331}=0.3, P=0.730
Lick & chew	T_{17}=-3.2, P=0.006	T_{332}=-0.5, P=0.649	T_{332}=-0.7, P=0.466	T_{331}=-1.3, P=0.188	T_{331}=-1.5, P=0.141

Table A3.4.1 Results of general (overall) analysis for direct observation state behaviours performed during interactive testing, using univariate (the effect of a single variable is considered) and multivariate (the effect of a number of variables and the interaction between these variables is considered) statistics.

Behaviour	Univariate Statistics			Multivariate Statistics	
	Experimental Group	Treatment	Time	Group*Treatment	Group*Time
Hindlimb weight-shift	T_{17}=-1.5, P=0.165	T_{332}=1.2, P=0.227	T_{332}=0.1, P=0.932	T_{331}=1.3, P=0.205	T_{331}=-1.2, P=0.244
Forelimb lift	T_{17}=1.2, P=0.249	T_{332}=0.6, P=0.524	T_{332}=-1.3, P=0.206	T_{331}=0.3, P=0.768	T_{331}=0.3, P=0.736
Hindlimb lift	T_{17}=1.8, P=0.096	T_{332}=0.9, P=0.394	T_{332}=-2.0, **P=0.043**	T_{331}=3.3, **P<0.001**	T_{331}=0.2, P=0.805
Tail flick	T_{17}=3.3, **P=0.005**	T_{332}=1.3, P=0.196	T_{332}=-1.1, P=0.272	T_{331}=-0.3, P=0.740	T_{331}=2.8, P=0.005
Skin twitch	T_{17}=-2.6, **P=0.018**	T_{332}=1.9, P=0.053	T_{332}=1.3, P=0.196	T_{331}=2.4, P=0.016	T_{331}=-1.3, P=0.184
Stamp	T_{17}=2.3, **P=0.032**	T_{332}=-1.8, P=0.066	T_{332}=1.9, P=0.055	T_{331}=0.5, P=0.641	T_{331}=0.9, P=0.345

Table A3.4.2 Results of occurrence analysis for direct observation event behaviours, for which square root transformation was insufficient to normalise the distribution of residuals, due to high numbers of zero values (no behaviour performed in sample).

Behaviour	Univariate Statistics			Multivariate Statistics	
	Experimental Group	Treatment	Time	Group*Treatment	Group*Time
Hindlimb weight-shift	$F_{1,17}$=7.9, **P=0.012**	$F_{1,260}$=4.7, **P=0.031**	$F_{1,260}$=0.0, P=0.871	$F_{1,259}$=7.1, **P=0.008**	$F_{1,259}$=2.9, P=0.090
Forelimb lift	$F_{1,17}$=8.9, **P=0.008**	$F_{1,277}$=18.9, **P>0.001**	$F_{1,20}$=2.7, P=0.114	$F_{1,276}$=7.5, P=0.007	$F_{1,276}$=3.4, P=0.066
Hindlimb lift	$F_{1,17}$=3.8, P=0.088	$F_{1,131}$=3.0, P=0.069	$F_{1,131}$=1.5, **P=0.227**	$F_{1,130}$=1.1, P=0.298	$F_{1,130}$=3.6, P=0.075
Tail flick	$F_{1,17}$=0.6, P=0.007	$F_{1,117}$=7.6, P=0.431	$F_{1,116}$=1.2, **P=0.269**	$F_{1,116}$=0.3, P=0.5661	$F_{1,116}$=1.1, P=0.304
Skin twitch	$F_{1,10}$=0.4, P=0.593	$F_{1,15}$=0.3, P=0.550	$F_{1,15}$=0.2, P=0.625	$F_{1,14}$=0.3, P=0.592	$F_{1,10}$=, P=0.963
Stamp	$F_{1,7}$=0.5, P=0.489	$F_{1,20}$=0.1, P=0.782	$F_{1,20}$=5.1, **P=0.036**	$F_{1,19}$=0.0, P=0.966	$F_{1,19}$=0.5, P=0.479

Table A3.4.3 Results of level of occurrence analysis for direction observation event behaviours, for which square root transformation was insufficient to normalise the distribution of residuals, due to high numbers of zero values (no behaviour performed in sample).

A3.5 EVOKED BEHAVIOUR RESULTS

Behaviour	Univariate Statistics			Multivariate Statistics	
	Experimental Group	Treatment	Time	Group*Treatment	Group*Time
Ears back	T_{17}=-5.5, **P<0.001**	T_{343}=7.9, **P<0.001**	T_{343}=-0.7, P=0.467	T_{342}=-7.3, **P<0.001**	T_{342}=-0.3, P=0.730
Head up (towards handler)	T_{17}=0.7, P=0.493	T_{343}=-2.5, **P=0.011**	T_{343}=0.6, P=0.578	T_{342}=-0.4, P=0.667	T_{342}=-0.1, P=0.960
Head up (away from handler)	T_{17}=-0.0, P=0.999	T_{343}=0.0, P=0.999	T_{343}=0.1, P=0.884	T_{342}=-0.0, P=0.999	T_{342}=0.0, P=0.999
Head up (straight)	T_{17}=0.1, P=0.886	T_{343}=0.4, P=0.681	T_{343}=-0.3, P=0.791	T_{342}=1.3, P=0.195	T_{342}=-0.7, P=0.463
Head down (towards handler)	T_{17}=-1.7, P=0.098	T_{343}=0.3, P=0.758	T_{343}=-0.6, P=0.551	T_{342}=-0.9, P=0.373	T_{342}=0.6, P=0.523
Head down (straight)	T_{17}=-0.8, P=0.435	T_{343}=3.9, **P<0.001**	T_{343}=-0.5, P=0.587	T_{342}=0.0, P=0.999	T_{342}=2.3, **P=0.025**
Exploratory	T_{17}=1.6, P=0.131	T_{343}=-4.2, **P<0.001**	T_{343}=1.2, P=0.242	T_{342}=1.9, P=0.062	T_{342}=0.0, P=0.983

Table A3.5.1 Results of general (overall) analysis for state behaviours performed during interactive testing, using univariate (the effect of a single variable is considered) and multivariate (the effect of a number of variables and the interaction between these variables is considered).

	Univariate Statistics			Multivariate Statistics	
	Experimental Group	**Treatment**	**Time**	**Group*Treatment**	**Group*Time**
Stamp	T_{17}=0.0, P=0.999	T_{343}=100.6, **P<0.001**	T_{343}=1.6, P=0.111	T_{342}=26.3, **P<0.001**	T_{342}=-0.0, P=0.999
Step Away	T_{17}=3.1, **P=0.007**	T_{343}=-4.3, **P<0.001**	T_{343}=1.0, P=0.320	T_{342}=2.4, **P=0.017**	T_{342}=-0.9, P=0.369
Tail Flick	T_{17}=1.4, P=0.178	T_{343}=0.2, P=0.848	T_{343}=-1.2, P=0.250	T_{342}=-61.6, **P<0.001**	T_{342}=-0.8, P=0.446
Forelimb lift	T_{17}=1.1, P=0.307	T_{343}=-1.3, P=0.199	T_{343}=-0.1, P=0.953	T_{342}=0.0, P=0.953	T_{342}=-0.1, P=0.277
Ear Flick	T_{17}=-2.2, P=0.999	T_{342}=-1.6, P=0.111	T_{343}=57.0, **P<0.001**	T_{342}=0.0, P=0.999	T_{342}=28.8, **P<0.001**
Head Movement	T_{17}=0.9, P=0.387	T_{343}=-0.3, P=0.737	T_{343}=-0.3, P=0.733	T_{342}=0.0, P=0.999	T_{342}=-0.5, P=0.603

Table A3.5.2 Results of occurrence analysis for event behaviours occurring during interactive testing, for which square root transformation was insufficient to normalise the distribution of residuals, due to high numbers of zero values (no behaviour performed in sample).

	Univariate Statistics			Multivariate Statistics	
	Experimental Group	**Treatment**	**Time**	**Group*Treatment**	**Group*Time**
Stamp	NA	NA	$F_{1,2}$=3.3, P=0.213	NA	NA
Step Away	$F_{1,13}$=3.2, P=0.095	$F_{1,22}$=1.5, P=0.235	$F_{1,22}$=0.7, P=0.402	$F_{1,12}$=1.2, P=0.302	$F_{1,21}$=0.4, P=0.560
Tail Flick	$F_{1,4}$=0.4, P=0.545	$F_{1,4}$=1.4, P=0.304	$F_{1,3}$=0.5, P=0.544	NA	$F_{1,2}$=0.6, P=0.522
Forelimb lift	$F_{1,3}$=0.0, P=0.178	$F_{1,3}$=0.0, P=0.178	NA	NA	NA
Ear Flick	NA	NA	NA	NA	NA
Head Movement	$F_{1,6}$=2.8, P=0.145	$F_{1,4}$=3.5, P=0.133	$F_{1,4}$=0.4, P=0.583	NA	$F_{1,3}$=0.2, P=0.694

Table A3.5.3 Results of level of occurrence analysis for event behaviours, occurring during interactive testing for which square root transformation was insufficient to normalise the distribution of residuals, due to high numbers of zero values (no behaviour performed in sample).'NA' indicates samples where insufficient data precluded statistical testing, due to lack of performance of behaviour

APPENDIX FOUR

CHAPTER FIVE RESULTS

A4.1 HORSE DETAILS

Experimental Group	Number	Sex	Age (y,m)	Breed	Weight (kg)	Onset of Condition	Possible precipitating /concurrent conditions
Laminitic	1	G	13,0	Connemara	480	1 month	Obese, increased liver enzymes
Laminitic	2	M	6,11	Welsh cob	460	2 months	Obese
Laminitic	3	M	20,9	Show Pony	300	Recurrent >1 year	Obese
Laminitic	4	M	6,9	Cob	490	2 days	None known
Laminitic	5	G	8,9	Welsh	298	Recurrent >1 year	Grain overload
Laminitic	6	S	11,11	Highland	574	Recurrent >1 year	Obese
Laminitic	7	G	13,11	Cob	560	Recurrent >1 year	Access to rich pasture
Control	1	G	10, 9	Thoroughbred X	420	-	-
Control	2	M	9, 7	Cob	450	-	-
Control	3	M	19,3	Welsh A	350	-	-
Control	4	M	8,1	Cob	470	-	-
Control	5	G	13, 2	Welsh	360	-	-
Control	6	S	14,0	Thoroughbred X	590	-	-
Control	7	G	15, 1	Cob	450	-	-

Table A4.1.1 Details of experimental animals.(**Sex:** G = gelding; S = stallion; M = mare)

A4.2 SPONTANEOUS BEHAVIOUR RESULTS

Behaviour	Univariate Statistics				Multivariate Statistics	
	Group	Time	Time of Day	Day	Group*Time	Group*Day
Inattentive (RH)	T_{12}=-0.74, P=0.472	T_{147}=-0.8, P=0.429	$F_{2,146}$=3.1, **P=0.046**	$F_{4,140}$=2.2, P=0.075	T_{146}=-0.1, P=0.940	$F_{4,140}$=0.0, P=0.999
Inattentive (ST)	T_{12}=-1.5, P=0.164	T_{147}=3.1, **P=0.003**	$F_{2,146}$=3.5, **P=0.033**	$F_{4,144}$=2.2, P=0.071	T_{146}=-1.0, P=0.317	$F_{4,140}$=0.0, P=0.999
Total lying	T_{12}=-2.6, **P=0.024**	T_{149}=1.0, P=0.344	$F_{2,148}$=4.4, **P=0.014**	$F_{2,146}$=2.1, P=0.08	T_{148}=-0.0, P=0.966	$F_{4,142}$=0.5, P=0.761
Head low	T_{12}=-2.2, **P=0.046**	T_{143}=0.3, P=0.738	$F_{2,142}$=6.3, **P=0.002**	$F_{4,140}$=0.4, P=0.798	T_{142}=0.7, P=0.471	$F_{4,136}$=0.4, P=0.810
Exploratory	T_{12}=0.3, P=0.774	T_{145}=-1.5, P=0.145	$F_{2,144}$=0.58, P=0.56	$F_{4,142}$=1.6, P=0.171	T_{144}=0.2, P=0.883	$F_{4,138}$=0.3, P=0.876
Stand	T_{12}=-0.3, P=0.804	T_{149}=-2.3, **P=0.020**	$F_{2,148}$=5.0, **P=0.008**	$F_{4,146}$=3.9, **P=0.005**	T_{148}=0.0, P=0.984	$F_{4,138}$=0.4, P=0.839
Rest hindlimb	T_{12}=4.9, **P<0.001**	T_{141}=2.5, **P=0.014**	$F_{2,140}$=2.8, P=0.064	$F_{4,138}$=3.6, **P=0.008**	T_{140}=-1.7, P=0.093	$F_{4,134}$=1.6 , P=0.186
Walk	T_{12}=2.4, **P=0.030**	T_{149}=-0.6, P=0.621	$F_{2,149}$=2.5, P=0.089	$F_{4,146}$=3.1, **P=0.019**	T_{148}=1.0, P=0.926	$F_{4,142}$=1.0, P=0.405
Lie sternally	T_{12}=-1.6, P=0.105	T_{149}=0.5, P=0.471	$F_{2,148}$=1.9, P=0.148	$F_{4,146}$=1.4, P=0.252	T_{148}=0.5, P=0.610	$F_{4,142}$=0.7, P=0.613
Lie laterally	T_{12}=-3.4, **P=0.006**	T_{149}=1.0, P=0.313	$F_{2,148}$=7.1, **P=0.001**	$F_{4,146}$=2.1, P=0.08	T_{148}=-0.6, P=0.527	$F_{4,142}$=0.4, P=0.803
Grooming	T_{12}=-0.8, P=0.467	T_{149}=1.5, P=0.129	$F_{2,148}$=0.4, P=0.693	$F_{2,140}$=1.3, P=0.288	T_{148}=3.0, **P=0.003**	$F_{4,142}$=1.5, P=0.203
Head up	T_{12}=2.2, **P=0.046**	T_{143}=-0.3, P=0.739	$F_{2,142}$=6.3, **P=0.002**	$F_{4,140}$=0.4, P=0.798	T_{142}=-0.7, P=0.471	$F_{4,136}$=0.4, P=0.810
Head down	T_{12}=-0.8, P=0.458	T_{143}=-0.2, P=0.835	$F_{2,142}$=1.2, P=0.304	$F_{4,140}$=0.9, P=0.491	T_{142}=-0.8, P=0.446	$F_{4,136}$=2.3, P=0.063
Head Level	T_{12}=-2.2, **P=0.049**	T_{143}=0.4, P=0.683	$F_{2,142}$=7.6, P<0.001	$F_{1,140}$=0.8, P=0.554	T_{142}=1.1, P=0.268	$F_{4,136}$=1.5, P=0.205
Front	T_{12}=2.0, P=0.064	T_{149}=-1.8, P=0.068	$F_{2,148}$=4.7, **P=0.011**	$F_{4,146}$=1.2, P=0.293	T_{148}=0.8 P=0.419	$F_{4,142}$=0.2, P=0.948
Middle	T_{12}=-0.1, P=0.897	T_{149}=2.9, **P=0.004**	$F_{2,148}$=0.9, P=0.422	$F_{4,146}$=1.3, P=0.283	T_{148}=-1.7, P=0.090	$F_{4,142}$=1.3, P=0.271
Back	T_{12}=-3.0, **P=0.011**	$F_{2,148}$=2.7,P=0.744	$F_{2,148}$=2.7, P=0.074	$F_{4,146}$=1.3, P=0.263	T_{148}=-0.0, P=0.996	$F_{4,142}$=0.1, P=0.975

Table A4.2.1 Results of general (overall) analysis for state behaviours using univariate (the effect of a single variable is considered) and multivariate (the effect of a number of variables and the interaction between these variables is considered) statistics. Group = laminitic or control, time = number of hours from beginning of the study, time of day = time of day at which sample was taken (0600h, 1400h or 2200h), day = day on which sample was taken (1-5).

Behaviour	Univariate Statistics				Multivariate Statistics	
	Experimental Group	**Time**	**Day**	**Time of Day**	**Group*Time**	**Group*Day**
Weight shift	$F_{1,12}$=13.9,**P=0.003**	$F_{1,141}$=3.7,P=0.056	$F_{1,138}$=1.0,P=0.410	$F_{2,140}$=7.4,**P<0.001**	$F_{1,140}$=0.4,P=0.552	$F_{4,142}$=0.2,P=0.194
Lift Forelimb	$F_{1,12}$=10.9,**P=0.006**	$F_{1,141}$=1.3,P=0.259	$F_{1,138}$=0.6,P=0.692	$F_{2,140}$=3.0,P=0.054	$F_{1,140}$=3.6,P=0.061	$F_{4,142}$=1.7,P=0.164
Head Shake	$F_{1,12}$=0.2, P=0.686	$F_{1,149}$=0.1,P=0.732	$F_{1,146}$=0.2,P=0.954	$F_{2,148}$=3.4,**P=0.037**	$F_{1,148}$=0.6,P=0.706	$F_{4,134}$=1.0,P=0.397

Table A4.2.2 Results of general (overall) analysis for event behaviours using univariate (the effect of a single variable is considered) and multivariate (the effect of a number of variables and the interaction between these variables is considered) statistics

A4.3 TIME POINT ANALYSIS

Behaviour	Univariate Statistics			Multivariate Statistics	
	Experimental group	Time	Day	Group*Time	Group*Day
Inattentive (RH)	T_{12}=-0.0, **P<0.001**	T_{33}=0.0, P=0.857	NA	T_{32}=0.0, **P<0.001**	NA
Inattentive (ST)	T_{12}=-0.0, **P<0.001**	T_{33}=0.0, **P<0.001**	NA	T_{32}=85.0, **P<0.001**	NA
Total lying	T_{12}=-2.3, **P=0.043**	T_{35}=-0.4, P=0.712	$F_{3,33}$=1.6, P=0.213	T_{35}=-0.4, P=0.546	$F_{3,30}$=0.5, P=0.671
Head low	T_{12}=-1.4, P=0.176	T_{31}=-0.5, P=0.613	$F_{3,29}$=1.2, P=0.327	T_{30}=0.1, P=0.930	$F_{3,26}$=0.2, P=0.890
Exploratory	T_{12}=-0.1, P=0.925	T_{33}=-2.5, P=0.016	$F_{3,31}$=2.3, P=0.094	T_{32}=1.4, P=0.180	NA
Stand	T_{12}=-0.5, P=0.636	T_{35}=-0.5, P=0.595	$F_{3,33}$=0.9, P=0.439	T_{34}=-0.1, P=0.940	$F_{3,30}$=0.0, P=0.999
Rest hindlimb	T_{12}=3.8, **P=0.002**	T_{33}=1.9, P=0.068	$F_{3,31}$=1.3, P=0.289	T_{32}=-1.6, P=0.114	$F_{3,28}$=1.8, P=0.171
Walk	T_{12}=2.4, **P=0.033**	T_{149}=-0.6, P=0.537	$F_{3,33}$=4.1, P=0.014	T_{34}=-1.5, P=0.141	$F_{3,30}$=0.8, P=0.500
Lie sternally	T_{12}=-1.7, P=0.112	T_{35}=-0.2, P=0.839	$F_{3,33}$=0.3, P=0.794	T_{34}=-1.0, P=0.323	$F_{3,33}$=2.4, P=0.083
Lie laterally	T_{12}=-2.3, P=**0.039**	T_{35}=-0.6, P=0.534	$F_{3,33}$=2.4, P=0.083	T_{34}=0.1, P=0.915	$F_{3,30}$=0.3, P=0.851
Grooming	T_{12}=-0.8, P=0.427	T_{35}=2.2, **P=0.029**	$F_{3,33}$=2.1, P=0.120	T_{34}=-1.6, P=0.129	$F_{3,30}$=1.2, P=0.340
Head up	T_{12}=1.4, P=0.176	T_{31}=0.5, P=0.613	$F_{3,29}$=1.2, P=0.327	T_{30}=-0.1, P=0.093	$F_{3,26}$=0.2, P=0.890
Head down	T_{12}=-2.2, **P=0.048**	T_{31}=1.3, P=0.205	$F_{3,29}$=1.4, P=0.273	T_{30}=-0.9, P=0.388	$F_{3,26}$=0.7, P=0.575
Head Level	T_{12}=-1.1, P=0.290	T_{31}=-0.8, P=0.415	$F_{3,29}$=1.3, P=0.309	T_{30}=0.1, P=0.887	$F_{3,26}$=0.3, P=0.820
Front	T_{12}=1.7, P=0.121	T_{35}=-0.0, P=0.992	$F_{3,33}$=0.5, P=0.712	T_{34}=0.1, P=0.903	$F_{3,30}$=1.8, P=0.162
Middle	T_{12}=-0.5, P=0.602	T_{35}=1.1, P=0.265	$F_{3,33}$=2.2, P=0.102	T_{34}=-0.6, P=0.541	$F_{3,30}$=2.7, P=0.064
Back	T_{12}=-1.8, P=0.092	T_{35}=-1.9, P=0.069	$F_{3,33}$=1.8, P=0.169	T_{34}=-0.1, P=0.945	$F_{1,12}$=2.1, P=0.172
Forelimb lifting	$F_{1,12}$=11.1, **P=0.006**	$F_{1,33}$=2.1, P=0.157	$F_{3,31}$=1.0, P=0.407	$F_{1,32}$=1.2, P=0.291	$F_{3,28}$=0.5 P=0.716

Table A4.3.1 Results of time point analysis at 0600h for state behaviours using univariate (the effect of a single variable is considered) and multivariate (the effect of a number of variables and the interaction between these variables is considered) statistics.

Behaviour	Univariate Statistics			Multivariate Statistics	
	Group	Time	Day	Group*Time	Group*Day
Weight shift	T_{12}=-3.0, **P=0.012**	T_{33}=0.2, P=0.809	$F_{3,31}$=0.2, P=0.880	T_{32}=0.5, P=0.612	$F_{3,28}$=0.1, P=0.958
Head shake	T_{12}=0.8, P=0.451	T=-0.1, P=0.892	$F_{3,33}$=0.4, P=0.750	T_{34}=0.4, P=0.675	$F_{3,30}$=0.4, P=0.712

Table A4.3.2 Results of occurrence analysis at 0600h. This test was used for event behaviours for which square root transformation was insufficient to normalise the distribution of residuals, due to high numbers of zero values (no behaviour performed in sample).

Behaviour	Univariate Statistics			Multivariate Statistics	
	Group	Time	Day	Group*Time	Group*Day
Weight shift	$F_{1,11}$=1.4, P=0.269	$F_{1,18}$=10.7, P=0.004	$F_{3,16}$=3.6, **P=0.04**	$F_{1,17}$=0.3, P=0.579	$F_{3,13}$=0.6, P=0.605
Head shake	$F_{1,11}$=1.7, P=0.214	$F_{1,14}$=0.6, P=0.453	$F_{3,12}$=1.0, P=0.420	$F_{1,13}$=4.6 P=0.051	$F_{3,9}$=1.6, P=0.251

Table A4.3.3 Results of level of occurrence analysis at 0600h. This test was used for event behaviours for which square root transformation was insufficient to normalise the distribution of residuals, due to high numbers of zero values (no behaviour performed in sample).'NA' indicates samples where insufficient data precluded statistical testing, due to lack of performance of behaviour.

Behaviour	Univariate Statistics			Multivariate Statistics	
	Experimental group	**Time**	**Day**	**Group*Time**	**Group*Day**
Inattentive (RH)	T_{12}=-0.6, P=0.579	T_{43}=-2.3, P=0.307	**$F_{4,40}$=4.1, P=0.007**	T_{42}=0.4, P=0.720	$F_{4,36}$=0.0, P=0.999
Inattentive (ST)	T_{12}=-0.3, P=0.787	T_{43}=5.2, **P<0.001**	NA	T_{42}=-0.0, P=0.999	NA
Total lying	T_{12}=-1.9, P=0.083	T_{43}=0.3, P=0.797	$F_{4,40}$=0.5, P=0.712	T_{42}=0.1, P=0.893	$F_{4,36}$=6.7, **P<0.001**
Head low	T_{12}=-2.5, **P=0.027**	T_{43}=0.6, P=0.523	$F_{4,40}$=0.4, P=0.815	T_{42}=0.0, P=0.985	$F_{4,36}$=0.4, P=0.812
Exploratory	T_{12}=0.9, P=0.388	T_{43}=0.2, P=0.851	NA	T_{42}=-7.5, **P<0.001**	NA
Stand	T_{12}=-0.2, P=0.810	T_{43}=-1.7, P=0.098	$F_{4,40}$=2.0, P=0.100	T_{42}=-0.3, P=0.803	$F_{4,36}$=0.2, P=0.947
Rest hindlimb	T_{12}=3.0, **P=0.011**	T_{38}=0.7, P=0.105	$F_{4,35}$=2.2, P=0.09	T_{37}=-0.6, P=0.563	$F_{4,31}$=0.4, P=0.820
Walk	T_{12}=1.2, P=0.251	T_{43}=-0.3, P=0.771	$F_{4,40}$=2.4, P=0.675	T_{42}=-1.1, P=0.283	$F_{4,36}$=3.3, **P=0.022**
Lie sternally	T_{12}=-0.7, P=0.510	T_{43}=-0.3, P=0.758	$F_{4,40}$=2.2, P=0.092	T_{42}=0.9, P=0.351	$F_{4,36}$=0.5, P=0.711
Lie laterally	T_{12}=-2.7, **P=0.019**	T_{43}0.9, P=0.351	$F_{4,40}$=0.9, P=0.46	T_{42}=-0.5, P=0.587	$F_{4,36}$=0.1, P=0.994
Grooming	T_{12}=0.4, P=0.700	T_{12}=1.0, P=0.347	$F_{4,40}$=0.6, P=0.643	T_{42}=3.0, **P=0.004**	$F_{4,36}$=0.1, P=0.994
Head up	T_{12}=2.5, **P=0.027**	T_{43}=-0.6, P=0.524	$F_{1,40}$=0.1, P=0.742	T_{42}=-0.0, P=0.986	$F_{4,36}$=0.4, P=0.812
Head down	T_{12}=0.3, P=0.737	T_{43}=-0.9, P=0.355	$F_{4,40}$=0.3, P=0.893	T_{42}=-2.2, P=0.061	$F_{4,36}$=2.5, P=0.058
Head Level	T_{12}=-2.7, **P=0.02**	T_{43}=1.1, P=0.298	$F_{4,40}$=0.7, P=0.566	T_{42}=1.2, P=0.244	$F_{4,36}$=1.7, P=0.169
Front	T_{12}=1.7, P=0.115	T_{43}=-0.7, P=0.460	$F_{4,40}$=0.1, P=0.971	T_{42}=-0.2, P=0.826	$F_{4,36}$=0.2, P=0.958
Middle	T_{12}=0.7, P=0.500	T_{43}=1.6, P=0.109	$F_{3,33}$=1.3, P=0.291	T_{42}=-0.3, P=0.789	$F_{4,36}$=1.6, P=0.183
Back	T_{12}=-1.7, P=0.107	T_{43}=-1.0, P=0.335	$F_{4,40}$=2.5, P=0.059	T_{42}=0.6, P=0.559	$F_{4,36}$=0.3, P=0.882
Forelimb lifting	$F_{1,12}$=16.0, **P=0.002**	$F_{1,38}$=0.0, P=0.950	$F_{3,31}$=0.3, P=0.862	$F_{1,37}$=1.3, P=0.270	$F_{4,31}$=1.0, P=0.401

Table A4.3.4 Results of time point analysis at 2200h for state behaviours using univariate (the effect of a single variable is considered) and multivariate (the effect of a number of variables and the interaction between these variables is considered) statistics.

Behaviour	Univariate Statistics			Multivariate Statistics	
	Group	Time	Day	Group*Time	Group*Day
Weight shift	T_{12}=-2.2, **P=0.048**	T_{38}=0.2, P=0.867	$F_{4,35}$=0.3, P=0.857	T_{37}=-1.4, P=0.158	$F_{4,31}$=0.1, P=0.976
Head shake	T_{12}=0.0, P=0.999	T_{43}=-2.0, P=0.054	$F_{4,40}$=1.3, P=0.278	T_{42}=-0.6, P=0.555	$F_{4,36}$=0.3, P=0.882

Table A4.3.5 Results of occurrence analysis at 2200h. This test was used for event behaviours for which square root transformation was insufficient to normalise the distribution of residuals, due to high numbers of zero values (no behaviour performed in sample).

Behaviour	Univariate Statistics			Multivariate Statistics	
	Group	Time	Day	Group*Time	Group*Day
Weight shift	$F_{1,12}$=1.3, P=0.285	$F_{1,28}$=1.6, P=0.215	$F_{4,25}$=0.5, P=0.709	$F_{1,27}$=-.2, P=0.698	$F_{4,21}$=0.4, P=0.803
Head shake	$F_{1,11}$=2.2, P=0.162	$F_{1,14}$=0.5, 0.482	$F_{4,11}$=0.3, P=0.882	$F_{1,13}$=0.0, P= 0.953	$F_{4,7}$=0.6, P=0.693

Table A4.3.6 Results of level of occurrence analysis at 2200h. This test was used for event behaviours for which square root transformation was insufficient to normalise the distribution of residuals, due to high numbers of zero values (no behaviour performed in sample).'NA' indicates samples where insufficient data precluded statistical testing, due to lack of performance of behaviour.

A4.4 DIRECT OBSERVATION SPONTANEOUS BEHAVIOUR RESULTS

Behaviour	Univariate Statistics			
	Group	Time	Am or Pm	Day
Attentive	T_{12}=-0.9, P=0.369	T_{95}=2.3, **P=0.021**	T_{95}=2.7, P=0.595	$F_{4,92}$=3.3, **P=0.019**
Ears forward	T_{12}=1.9, P=0.077	T_{95}=-1.5, P=0.133	T_{95}=2.6, **P=0.01**	$F_{4,92}$=0.7, P=0.58
Ears back	T_{12}=-0.3, P=0.796	T_{95}=3.7, **P<0.001**	T_{95}=-3.5, **P<0.001**	$F_{4,92}$=5.0, **P=0.001**
Ears side	T_{12}=-1.2, P=0.265	T_{95}=-1.7, P=0.097	T_{95}=1.3, P=0.210	$F_{4,92}$=1.8, P=0.140
Lick & chew	T_{12}=-0.3, P=0.745	T_{95}=0.5, P=0.600	T_{95}=1.1, P=0.262	$F_{4,92}$=2.5, **P=0.046**

Table A4.4.1 Results of general (overall) analysis for direct observation state behaviours performed during interactive testing, using univariate (the effect of a single variable is considered) statistics.

Behaviour	Multivariate Statistics		
	Group*Time	Group*AmPm	Group*Day
Attentive	T_{94}=-1.3, P=0.198	T_{94}=-0.0, P=0.995	$F_{4,88}$=1.3, P=0.267
Ears forward	T_{94}=-0.1, P=0.893	T_{94}=1.3, P=0.191	$F_{4,88}$=0.6, P=0.663
Ears back	T_{94}=-0.8, P=0.416	T_{94}=-0.5, P=0.852	$F_{4,88}$=0.5, P=0.759
Ears side	T_{94}=0.7, P=0.474	T_{94}=-0.6, P=0.560	$F_{4,88}$=0.2, P=0.945
Lick & chew	T_{94}=-1.1, P=0.292	T_{94}=1.0, P=0.317	$F_{4,88}$=0.9, P=0.118

Table A4.4.2 Results of general (overall) analysis for direct observation state behaviours performed during interactive testing, using multivariate (the effect of a number of variables and the interaction between these variables is considered) statistics.

Behaviour	Univariate Statistics			
	Group	Time	AmPm	Day
Hindlimb weight-shift	T_{12}=-1.6, P=0.146	T_{95}=0.5, P=0.302	T_{95}=-1.5, P=0.144	$F_{4,92}$=1.4, P=0.229
Forelimb lift	T_{12}=-1.5, P=0.154	T_{95}=0.1, P=0.955	T_{95}=1.0, P=0.309	$F_{4,92}$=0.5, P=0.742
Hindlimb lift	T_{12}=0.6, P=0.565	T_{95}=-0.1, P=0.950	T_{95}=2.0, P=0.050	$F_{4,92}$=0.3, P=0.867
Tail flick	T_{12}=-0.4, P=0.724	T_{95}=0.3, P=0.750	T_{95}=-4.0, **P<0.001**	$F_{4,88}$=0.6, P=0.647
Skin twitch	T_{12}=-0.7, P=0.493	T_{95}=1.1, P=0.285	T_{95}=-2.7, **P=0.004**	$F_{4,30}$=3.8, P=0.055

Table A4.4.3 Results of occurrence analysis for direct observation event behaviours using multivariate statistics. These tests were used for behaviours for which square root transformation was insufficient to normalise the distribution of residuals, due to high numbers of zero values (no behaviour performed in sample).

Behaviour	Univariate Statistics			
	Group	Time	AmPm	Day
Hindlimb weight-shift	$F_{1,11}$=2.5, P=0.144	$F_{4,46}$=1.6, P=0.923	$F_{1,49}$=0.1, P=0.729	$F_{4,46}$=1.6, P=0.190
Forelimb lift	$F_{1,12}$=14.9, P=0.28	$F_{4,75}$=0.3, **P=0.002**	$F_{1,78}$=0.7, P=0.717	$F_{4,88}$=0.4, P=0.779
Hindlimb lift	$F_{1,12}$=3.6, P=0.081	$F_{4,49}$=1.5, P=0.161	$F_{1,52}$=4.5, **P=0.038**	NA
Tail flick	$F_{1,12}$=0.0, P=0.874	$F_{4,49}$=0.6, P=0.461	$F_{1,37}$=13.9, **P<0.001**	$F_{4,34}$=0.6, P=0.633
Skin twitch	$F_{1,5}$=0.3, P=0.625	$F_{4,13}$=0.0, P=0.870	$F_{1,16}$=4.2, P=0.057	$F_{3,29}$=2.1, P=0.128

Table A4.4.4 Results of level of occurrence analysis for direction observation event behaviours using univariate statistics. These tests were used for behaviours for which square root transformation was insufficient to normalise the distribution of residuals, due to high numbers of zero values (no behaviour performed in sample)

Behaviour	Multivariate Statistics		
	Group*Time	Group*AmPm	Group*Day
Hindlimb weight-shift	$F_{4,42}$=0.3, P=0.467	$F_{1,48}$=0.5, P=0.479	$F_{4,42}$=0.3, P=0.862
Forelimb lift	$F_{4,71}$=1.5, P=0.643	$F_{1,77}$=1.3, 0.260	$F_{4,92}$=0.3, P=0.80
Hindlimb lift	$F_{4,45}$=0.9, P=0.195	$F_{1,51}$=4.4, P=0.041	$F_{4,45}$=0.9, P=0.456
Tail flick	$F_{1,36}$=0.1, P=0.761	$F_{1,36}$=0.2, P=0.699	$F_{4,30}$=0.8, P=0.541
Skin twitch	$F_{1,15}$=0.2, P=0.658	$F_{1,15}$=0.2, P=0.664	$F_{3,26}$=0.2, P=0.913

Table A4.4.5 Results of level of occurrence analysis for direction observation event behaviours using multivariate statistics. These tests were used for behaviours for which square root transformation was insufficient to normalise the distribution of residuals, due to high numbers of zero values (no behaviour performed in sample).

A4.5 EVOKED BEHAVIOUR RESULTS

Behaviour	Univariate Statistics		Multivariate Statistics
	Group	Day	Group*Day
Ears back	T_{12}=1.1, P=0.299	$F_{4,26}$=0.7, P=0.615	$F_{4,22}$=1.2, P=0.344
Head up – turn towards handler	T_{11}1.6, P=0.142	$F_{4,26}$=0.4, P=0.793	$F_{4,22}$= 0.6, P=0.672
Head up – turn away from handler	T_{11}=-0.9, P=0.391	$F_{4,26}$=1.1, P=0.382	$F_{4,22}$=0.0, P=0.999
Head up - straight	T_{11}=-1.1, P=0.313	$F_{4,26}$=0.6, P=0.676	$F_{4,22}$=0.7, P=0.610
Head down – turn towards handler	T_{11}=0.4, P=0.733	$F_{4,26}$=0.8, P=0.535	$F_{4,22}$=0.0 , P=0.999
Head down - straight	T_{11}=-0.0, P=0.860	$F_{4,26}$=0.7, P=0.588	$F_{4,22}$=0.4 , P=0.799
Head down – turn away from handler	T_{11}=0.2, P=0.999	NA	NA
Exploratory	T_{11}=0.7, P=0.487	$F_{4,26}$=1.5, P=0.245	$F_{4,22}$=0.7, P=0.602

Table A4.5.1 Results of general (overall) analysis for state behaviours performed during interactive testing, using univariate (the effect of a single variable is considered) and multivariate (the effect of a number of variables and the interaction between these variables is considered).

Behaviour	Univariate Statistics		Multivariate Statistics
	Experimental Group	Day	Group*Day
Step Away	T_{11}=-0.1, P=0.924	$F_{3,18}$=1.8, P=0.164	$F_{4,23}$=0.1, P=0.972
Tail Flick	T_{11}=0.0, P=0.999	NA	NA
Forelimb lift	0.514	$F_{4,27}$=2.6, P=0.06	$F_{4,23}$=0.0, P=0.995

Table A4.5.2 Results of occurrence analysis for event behaviours occurring during interactive testing, for which square root transformation was insufficient to normalise the distribution of residuals, due to high numbers of zero values (no behaviour performed in sample). 'NA' indicates samples where insufficient data precluded statistical testing, due to lack of performance of behaviour.

Behaviour	Univariate Statistics		Multivariate Statistics
	Experimental Group	Day	Group*Day
Step Away	$F_{1,9}$=1.5, P=0.250	$F_{4,4}$=8.4, **P=0.032**	NA
Tail Flick	NA	NA	NA
Forelimb lift	$F_{1,3}$=0.4, P=0.587	NA	NA

Table A4.5.3 Results of level of occurrence analysis for event behaviours, occurring during interactive testing for which square root transformation was insufficient to normalise the distribution of residuals, due to high numbers of zero values (no behaviour performed in sample).'NA' indicates samples where insufficient data precluded statistical testing, due to lack of performance of behaviour.